MASTER OF SCIENCE

Full Field Laser Perfusion Imaging and Post Occlusive Reactive Hyperaemia in the Skin Microcirculation
A Biomarker of Cardiovascular Disease Risk?

Adams, Fiona

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Full Field Laser Perfusion Imaging and Post Occlusive Reactive Hyperaemia in the Skin Microcirculation: A Biomarker of Cardiovascular Disease Risk?

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Declaration

"I declare that the content of this project report is my own work, unless otherwise stated, and has not previously been submitted for any other assessment. The report is written in my own words and conforms to the University of Dundee's policy on plagiarism and academic dishonesty. Unless otherwise indicated, I have consulted all of the references cited in this report"

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Abstract

The full field laser perfusion imager (FLPI) is a new, non-invasive device which can measure dynamic changes in blood flow of the skin microcirculation. Endothelial dysfunction is the earliest detectable functional indicator of cardiovascular disease (CVD), contributing to the development of atherosclerosis. Assessment of endothelial function provides a powerful method to identify patients at risk of developing CVD before the appearance of clinical symptoms. FLPI in combination with post occlusive reactive hyperaemia (PORH) has the potential to become an important tool for the assessment of skin microvascular function and dysfunction. The aims of this project were to firstly develop a test of PORH (following 5 minutes of arterial occlusion) using FLPI and secondly to compare this test with the EndoPAT device, the only food and drug administration (FDA) validated assessment.

FLPI was used to assess the reproducibility of changes in forearm skin blood flow following PORH using different protocols to find the optimal settings for repeated measures (cuff position, measurement site and skin temperature). The most reproducible PORH results were found with an upper arm blood pressure cuff and a distal measurement site at the forearm. An upper arm cuff and a proximal measurement site at the forearm was second and a lower arm cuff and a distal measurement site the least reproducible (Variance *n*=10: 8%; *n*=10 17%; *n*=5 31%, respectively).

Skin heating was introduced to the PORH protocol to see how this affected the reproducibility of the test. Forearm skin heating to 35°C prior to and during PORH did not improve reproducibility of the test (*n*=10; Variance 41% vs. 8% without heating). The results from the developmental phase of the study indicate that the most reproducible method of PORH to use with FLPI is with an upper arm blood pressure cuff and a distal measurement site without heating of the forearm skin.

The most reproducible PORH protocol was then applied to two groups of healthy volunteers (G1 *n*=15: 18-30 years; G2 *n*=15: 40-70 years) to investigate the effect of age on endothelial function and to compare with the EndoPAT device, an alternative method of endothelial function assessment.

No significant differences were noted between G1 and G2 for the PORH response measured by FLPI (G1 228±74% vs. G2 230±86%) however a significant negative correlation was found between PORH response and age in G2 ($r=-0.599$; $p<0.05$). Significant differences in endothelial function were observed between G1 and G2 by EndoPAT (G1 2.68±0.6units vs. G2 2.28±0.6units, $p<0.05$). The PORH test also detected significant differences between males and females (M 202.1±63.5% vs. F 256.9±85.8%, $p<0.05$), across all study volunteers, which were undetected by EndoPAT.

PORH coupled with FLPI has the potential to become a useful biomarker of skin microvascular endothelial function. FLPI was able to detect changes in endothelial function between males and females and within an older healthy population. Further work is needed to evaluate this method in patients with varying levels of disease.

Abbreviations

ABI	ankle brachial pressure index
ACE	angiotensin-converting-enzyme
ACH	acetylcholine
AGE	advanced glycation end products
AIx	augmentation index
AIx@75	augmentation index standardised to a heart rate of 75 beats per minute
AUC	area under the curve
BH_4	tetrahydrobiopterin
BHF	British Heart Foundation
BMI	body mass index
BP	blood pressure
CAD	coronary artery disease
cGMP	cyclic guanosine 3', 5-monophosphate
CHD	coronary heart disease
CRP	C-reactive protein
CVC	cutaneous vascular conductance
CVD	cardiovascular disease

ECG	electrocardiogram
EDHF	endothelium-derived hyperpolarising factor
eNOS	endothelial nitric oxide synthase
FAD	flavin adenine dinucleotide
FDA	Food and Drug Administration
FLPI	full field laser perfusion imager
FMD	flow mediated dilatation
G1	group 1
G2	group 2
GC	guanylate cyclase
FHS	Framingham Heart Study
FRS	Framingham Risk Score
HDL	high density lipoprotein
IL-1	interleukin-1
IL-6	interleukin-6
LDI	laser Doppler imaging
LDF	laser Doppler flowmetry
LDL	low density lipoprotein

LSCI	laser speckle contrast imaging
MAP	mean arterial pressure
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NO	nitric oxide
NOS	nitric oxide synthase
PAT	peripheral arterial tonometry
PGI_2	prostaglandins
PORH	post occlusive reactive hyperaemia
PU	perfusion units
PWA	pulse wave analysis
RHI	reactive hyperaemia index
ROS	reactive oxygen species
SIMD	Scottish Index of Multiple Deprivation
SNP	sodium nitroprusside
THR	total hyperaemic response
TNF-α	tumor necrosis factor alpha
VOP	venous occlusion plethysmography
WHO	World Health Organisation

Chapter 1

Introduction

The introduction will firstly address cardiovascular disease (CVD) risk where traditional CVD risk factors will be considered alongside the Framingham Heart Study (FHS) and its subsequent risk model. Additionally, other established risk models, and the emergence of newer CVD risk factors will be discussed. A major regulator of CV function, the endothelium, will be addressed in terms of its physiological function under basal conditions paying particular attention to nitric oxide (NO), the effects of CVD risk factors on endothelial function and the consequences of endothelial dysfunction. The assessment of endothelial function will then be reviewed detailing the current techniques used to measure endothelial function including both invasive and non-invasive methods. The final section will summarise the clinical implications of endothelial dysfunction, before the aims and hypotheses of the research project are described.

1.1 Cardiovascular Disease

CVD, or diseases of the heart and circulatory system, are the leading cause of death worldwide, and their primary prevention is a global health priority. The latest statistics released by the World Health Organisation (WHO) reveal an estimated 17.3 million people died from CVD in 2008, accounting for just under one third of deaths worldwide; of these deaths, 7.3 million resulted from coronary heart disease (CHD) and 6.2 million from stroke (WHO, 2011). CVD continues to cause the greatest number of

deaths in the UK, despite a continuing decline in mortality rates. The most recent figures reveal almost 180,000 deaths were attributed to CVD in 2010, with around 80,000 deaths from CHD and 49,000 from stroke (BHF, 2012).

At the beginning of the 20th century, there were no known treatments for CVD that were able to prolong life and significant changes in CVD mortality were observed, with many western countries recording increases in CVD mortality. There was also limited knowledge of the actual cause of the disease process, making it extremely difficult to reduce CVD mortality rates. It is now understood that the beginning of CVD can be delayed by preventative measures, which can result in a marked increase in life expectancy. To develop preventative measures it is necessary to address preventable and modifiable pre-disposing factors, and because CVD is a multifactorial disease and progresses with time, the best approach was to conduct a longitudinal study to observe these factors and the disease state over an extended period of time (D'Agostino et al., 2013). This led way to the Framingham Heart Study (FHS), the largest epidemiological CVD study to date, with the aim of identifying common factors that contribute to CVD.

1.2 Cardiovascular Disease Risk

The FHS initially recruited a population of 5209 individuals (2336 men and 2873 women) aged between 30 and 62 years from the town of Framingham, Massachusetts who were free from overt symptoms of CVD and who had not previously suffered from a stroke or heart attack (Dawber, 1980). An additional offspring cohort was added in 1971 and a third generation cohort in 2001. The FHS has provided an invaluable insight not only into major CV risk factors including hypertension and hypercholesterolaemia, but also how their interactions over time lead to the development of CVD.

1.2.1 Cardiovascular Disease Risk Scores

The FHS paper, published on "Factors of Risk" (Kannel et al., 1961), provided evidence that CV risk factors occur prior to the development of overt disease and are associated with an increased risk of CVD development. The FHS went on to report risk equations, to provide a greater understanding of CVD risk assessment by identifying an individual's risk of developing CVD based on multiple risk factors. The Framingham Risk Score (FRS) was developed following multivariate analysis and at present is the most commonly used tool within North America to assess CVD risk (Davis, 2010). The risk of developing CVD is strongly influenced by the following combination of risk factors: age, gender, total cholesterol, high-density lipoprotein cholesterol, smoking behaviour, diabetes status, systolic blood pressure (BP) and treatment for hypertension (Jackson, 2000), all of which are incorporated into the FRS to calculate an individual's 10 year risk of developing CHD.

The FHS has made an unquestionable impact on the current understanding of CV risk factors and how they associate and contribute towards the development of overt CVD. However, the FRS, a product of the FHS, does come with limitations. Questions have been raised as to whether the results can be transferred to other populations, particularly different ethnic groups. Women may also be underserved using the FRS (D'Agostino et al., 2001, Michos et al., 2006). The FRS is able to identify individuals at high-risk of developing CVD, but young individuals with few CV risk factors may be overlooked and mistakenly given a low-risk score. The FRS is based on a 10 year risk prediction but offering a longer-term CVD risk assessment would potentially allow for risk factor adaptation at an earlier time point and provide improved prevention. A 30 year risk prediction model has also been generated to address this need, although a lifetime risk prediction model would likely prove more valuable. Individuals able to sustain an optimal risk profile until 50 years of age will have a significantly lower risk of CVD in

later life (Figure 1.1), emphasising the importance of early prevention (Lloyd-Jones et al., 2006).

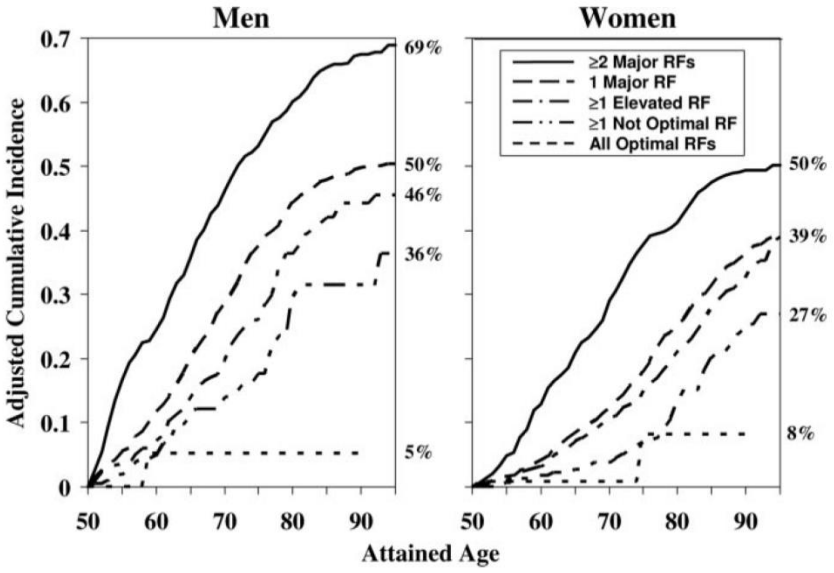
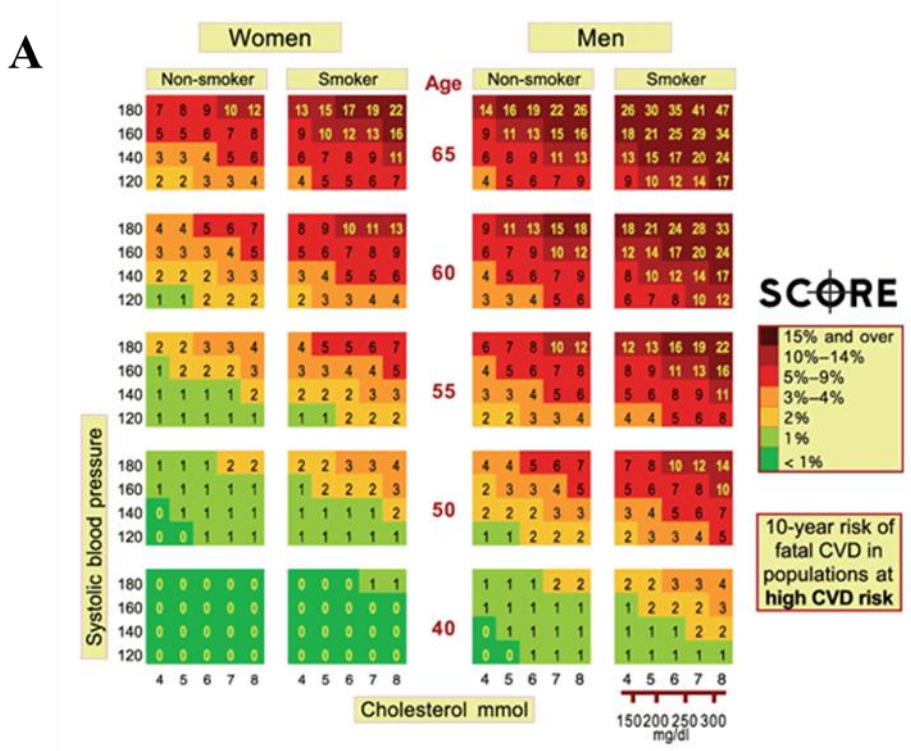


Figure 1.1 Cumulative incidence of CVD adjusted for the competing risk of death for men and women according to aggregate risk factor (RF) burden at 50 years of age. The percentages at the right of each graph correspond to the adjusted cumulative incidence to 95 years of age or lifetime risk of CVD. Optimal risk factors: total cholesterol <4.65 mmol/L, BP <120/80 mm Hg, non-smoker, and non-diabetic. Not optimal risk factors: total cholesterol 4.65-5.15 mmol/L, systolic BP 120-139 mm Hg, diastolic DP 80-89 mm Hg, non-smoker, and non-diabetic. Elevated risk factors: total cholesterol 5.16 -6.19 mmol/L, systolic BP 140-159 mm Hg, diastolic BP 90 to 99 mm Hg, non-smoker, and non-diabetic. Major risk factors: total cholesterol ≥6.20 mmol/L, systolic BP≥160 mm Hg, diastolic BP≥100 mm Hg, smoker, and diabetic. Circulation by American Heart Association reproduced with permission of Lippincott Williams & Wilkins (Lloyd-Jones et al., 2006) via Copyright Clearance Centre.

A number of other risk prediction models have been developed as a result of the FRS from different cohorts throughout the USA and Europe including SCORE, ASSIGN, PROCAM (Thakur et al., 2010), CURORE (Reriani et al., 2010), QRISK and Reynolds (Li et al., 2010). It is not within the scope of this review to detail each of these risk scores, but a selected few will be addressed; SCORE (Systematic COronary Risk Evaluation), QRISK and ASSIGN (Assessing Cardiovascular risk using the Scottish Intercollegiate Guidelines Network).

SCORE, which combined data from twelve European countries, was set up to develop a risk scoring system relevant to European populations. The study population was made up of a total of 205,178 participants recruited between the 1960's and 1980's and were

followed for 2.7 million person-years. A 10 year risk of fatal CVD was calculated but age was not recognised as a risk factor, instead it was used as a measure of exposure to risk. Other equations were calculated for coronary and non-coronary CVD death and for both high and low risk regions of Europe, using total cholesterol and the total cholesterol/high density lipoprotein (HDL) ratio (Figure 1.2). It was important that a risk model specific to the European population was established after it was concluded that models like the FRS failed to accurately calculate risk for this population; the FRS is based on an American population viewed to have higher risk factor treatment levels than European populations where a Mediterranean diet is popular and likely to affect level of risk. One of the advantages of the SCORE risk tool is that it is able to distinguish between low and high risk groups in European countries but some data still suggests that in certain countries, for example Germany, the model may overestimate an individual's risk (Neuhauser et al., 2005).



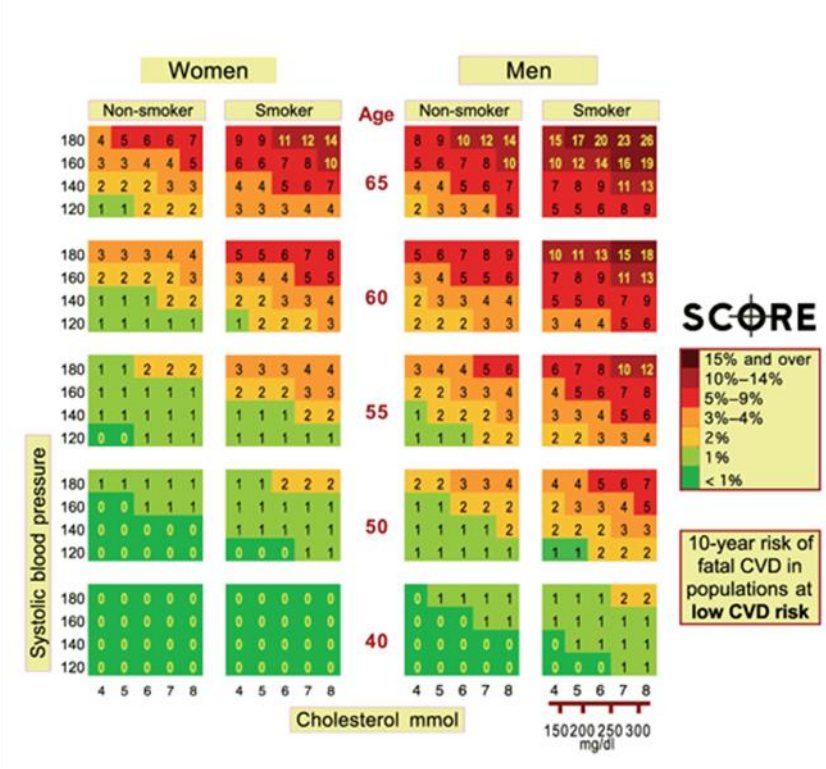


Figure 1.2 SCORE European Risk Charts: Ten year risk of fatal CVD in men and women with (**A**) high and (**B**) low CVD risk (based on total cholesterol) (European Society of Cardiology).

QRISK is a risk model designed specifically for the UK based on the QRESEARCH database which contains patient health records, obtained at general practices between January 1995 and April 2007, of 1.28 million people aged between 35 and 74 years who were free of CVD and diabetes. The initial risk score, QRISK1, measured age, sex, body mass index (BMI), smoking status, diabetes, total cholesterol to HDL cholesterol ratio, deprivation index, family history of CHD and use of antihypertensive treatment to predict the 10 year risk of developing CVD. Following evaluation, QRISK1 was found to be a better predictor of CVD risk in the UK population compared with the FRS and the model also presented a different high risk group of patients than the FRS with one in every ten patients reassigned into high or low risk categories (Hippisley-Cox et al., 2007). A subsequent risk model, the QRISK2, studied 1.58 million people and took into account ethnicity and chronic disease history, along with the factors used in the QRISK1 model, to calculate the 10 year risk of developing CVD. These factors were

not previously considered by the FRS and provide the opportunity for better risk stratification for individual patients to reduce health inequalities.

The ASSIGN risk score is derived from a study of a community in Scotland across twenty five districts. Study participants consisted of 6540 males and 6757 females free of CVD who were recruited in the 1980's and monitored up until March 1997. The 10 year risk of CVD events was determined by a number of factors including sex, age, smoking status, systolic BP, total cholesterol, HDL cholesterol, diabetes and for the first time family history of CHD as well as social deprivation scores based on area of residence using the Scottish Index of Multiple Deprivation (SIMD). The major advantage of this model over conventional risk scores is the inclusion of social deprivation; those people who were found to be more socially deprived by residence postcode were identified as higher risk and a positive family history of CHD also resulted in a high risk score (Woodward et al., 2007). Other risk score models, including FRS, are unable to differentiate between levels of social deprivation.

Despite the existence of many CVD risk models there is still evidence to suggest that these models fail to recognise a large proportion of individuals who are at risk of developing disease; more than fifty per cent of people diagnosed with coronary artery disease (CAD) do not have any traditional risk factors (Futterman and Lemberg, 1998). Furthermore, in a population of 136,905 patients who were hospitalised with CAD, almost half had normal levels of low density lipoprotein (LDL) cholesterol (Sachdeva et al., 2009), a well-known risk factor of CVD. In the current risk models these individuals would be mistakenly classified into a low risk group. Thus it is evident that other methods of risk assessment need to be considered to improve current risk assessments and help identify those who will develop CAD, even when classical risk factors are absent. A new approach may need to include revised recommended ranges for

parameters, such as LDL cholesterol, and newer CVD risk factors not currently incorporated into CVD risk models to improve risk classifications in those who have previously been overlooked.

1.3 New Cardiovascular Disease Risk Factors

Current risk score models are based on the traditional CVD risk factors that were first discovered as a result of the FHS, but more recently attention has turned to non-traditional risk factors including adiposity, oxidative stress and inflammation which also play an important role in the development of CVD (Figure 1.3).

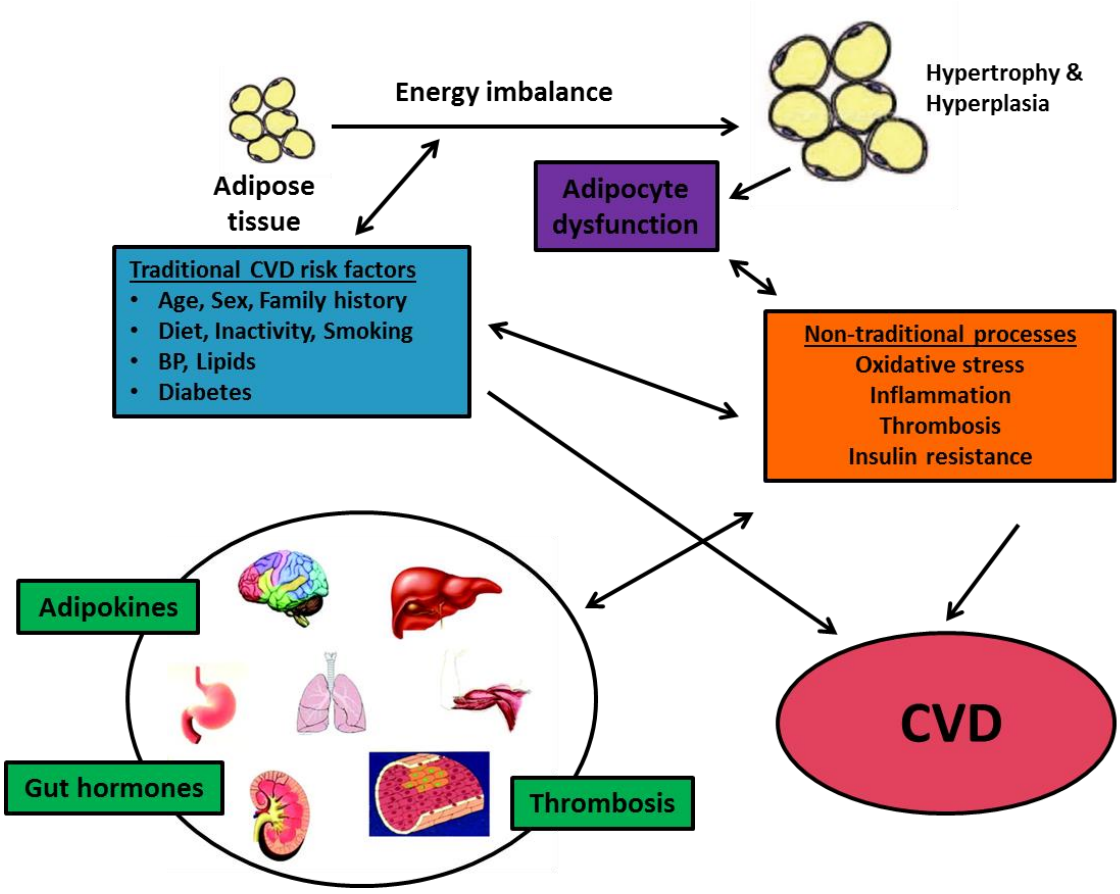


Figure 1.3 Overview of the pathophysiology of CVD including newer, non-traditional risk factors in addition to traditional risk factors. Adapted from Balagopal et al. (Balagopal et al., 2011).

The number of individuals who are overweight or obese continues to rise in the developed world. This can be attributed to lifestyle - sedentary behaviour with low

levels of physical activity and an excessive caloric intake - and inherited genes. Obesity, particularly the central or visceral type, is a chronic metabolic disorder associated with important CV comorbidities such as hypertension and atherosclerosis. Adipose tissue and adipocytes act as an endocrine organ and play a fundamental part in the pathogenesis of obesity. A wide range of bioactive substances known as adipokines, (leptin, adiponectin, resistin etc.), are released by the adipose tissue to help regulate numerous metabolic functions including glucose and lipid metabolism. In a state of obesity, the secretion of adipokines is altered and thought to cause an increased risk of obesity related CV disorders. For example, adiponectin which is anti-inflammatory, anti-atherogenic and has cardio-protective effects is down-regulated in obesity while leptin which is pro-inflammatory, pro-thrombotic and pro-oxidant is up-regulated and makes an important contribution to obesity related atherogenesis (Katagiri et al., 2007). The major consequence of adipocyte dysfunction is local inflammation, defined by an infiltration of inflammatory cells and increases in pro-inflammatory cytokines, a central process throughout atherosclerosis.

Following increasing evidence from animal model studies and human studies, it has emerged that an over production of reactive oxygen species (ROS) are crucial in the pathogenesis and development of CVD including dyslipidaemia, hypertension, atherosclerosis and heart failure (Heitzer et al., 2001). ROS are produced by several important oxidase enzymes including nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase, xanthine oxidase, uncoupled endothelial NO synthases and the mitochondrial electron transport through vascular smooth muscle cells, endothelial cells and adventitial cells. Oxidative stress (the imbalance between production of ROS and anti-oxidant defences) activates numerous signalling pathways leading to the activation of inflammation, cell proliferation, hypertrophy and apoptosis.

Numerous studies, both pre-clinical and clinical, have assessed the effect of anti-oxidants, such as vitamin E, on reducing ROS bioavailability but as yet there is no evidence to suggest that anti-oxidant supplements improve CV morbidity and mortality. The ideal anti-oxidant therapy appears to come from agents that can simultaneously stimulate NO production and inhibit ROS production, such as statins and angiotensin-converting-enzyme (ACE) inhibitors, both of which possess such properties. In addition to lipid lowering, statins are able to decrease ROS generated by NADPH oxidase and activate NOS (Cangemi et al., 2008). Statins have also been shown to improve vascular function, specifically endothelial function of the forearm, just two weeks after treatment started (John et al., 2001). The Study to Evaluate Carotid Ultrasound in patients treated with Ramipril and Vitamin E (SECURE), a sub study of the Heart Outcomes Prevention Evaluation Study (HOPE), demonstrated that treatment with the ACE inhibitor Ramipril has a beneficial effect on the progression of atherosclerosis, demonstrated by a decrease in the incidence of stroke, myocardial infarction and death in high risk patients, but there was no beneficial effect on disease progression with vitamin E. At present oxidative stress is not measured clinically however it remains a striking target not only for CVD prevention but also CVD therapy.

Inflammation is another known risk factor contributing to CVD which occurs as a result of oxidative stress. Inflammation plays a pivotal role in CVD with the inflammatory cascade proving specifically important throughout the stages of atherosclerosis (including plaque development, disruption and thrombosis). Pro-inflammatory risk factors such as injury and oxidised LDL cholesterol initiate the inflammatory response leading to the production of pro-inflammatory cytokines in the arterial wall. Primary pro-inflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α), mediate the attraction and migration of inflammatory cells into the vascular tissue. These cytokines also promote the release of messenger cytokines, like

IL-6, which cause acute phase reactants such as C-reactive protein (CRP) to be released into the circulation. The chronic inflammatory response ultimately results in the formation of a vulnerable plaque which is susceptible to rupture and thrombosis (Figure 1.4).

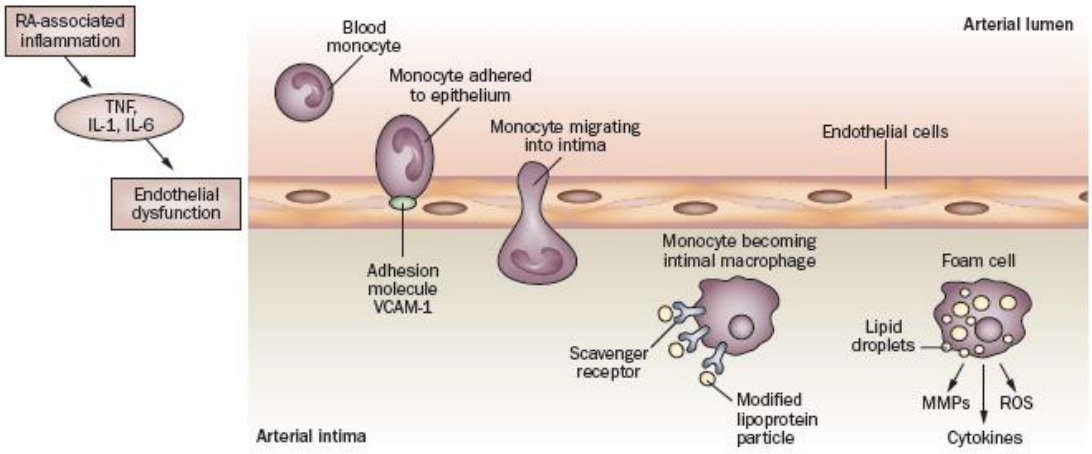


Figure 1.4 The inflammatory cascade in atherosclerosis. Endothelial cells can be activated by inflammation resulting in elevated expression of leukocyte adhesion molecules such as VCAM-1 and ICAM-1. VCAM-1 is responsible for the movement of monocytes towards the vessel wall. Monocytes that have adhered to the vessel wall travel to the intima, the inner lining of the blood vessel. Monocytes become macrophages at the intima and bind oxidised LDL, through scavenger receptors, and thereby become foam cells. Fatty streaks develop due to the continued accumulation of lipids and foam cells. Pro-inflammatory cytokines and ROS are produced by the foam cells, leading to increased recruitment of macrophages and T-cells. RA=rheumatoid arthritis; TNF=tumor necrosis factor; IL=interleukin; ICAM-1= intercellular adhesion molecule-1; VCAM-1=vascular cell adhesion molecule-1; ROS=reactive oxygen species; MMP=matrix metalloproteinase. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Rheumatology (Khan et al., 2010) copyright 2010 via Copyright Clearance Centre.

Clinical studies have highlighted strong relationships between markers of inflammation and the risk of future CV events. The inflammatory marker most readily used in clinical studies, due to its reliability and accessibility, is high sensitivity CRP which has been shown to be a good predictor of myocardial infarction and stroke, even better than LDL cholesterol (Ridker et al., 2002). However, CRP has the greatest value when used for primary prevention to identify individuals who are at high risk but are regarded as healthy (Ridker et al., 2002).

Since the identification of CV risk factors considerable advancements have been made in the prevention and treatment of CVD. These risk factors have been used to develop risk models which are essential tools in the management of the disease and capable of calculating a person's risk of developing CVD. The application of risk models provides a means of CVD risk stratification allowing those at highest risk of CVD to be easily distinguished from those at low and medium risk. More recently additional risk factors have emerged, such as oxidative stress and inflammation, which are already proving to have a key involvement in the disease process. To better understand the development and progression of CVD it is important that the underlying pathophysiological mechanisms underlying it are addressed, in particular the function of the endothelium, which is now known as a major regulator of CV function.

1.4 The Endothelium

The pivotal location of the endothelium allows the paracrine organ to perform a diverse range of functions within the body, and being situated within the inner lining of blood vessels makes it ideally suited to fulfil its role as a "barometer" of vascular health (Herrmann and Lerman, 2008). Once just thought of as an interface between blood and the vessel wall, the endothelium is now known to sense and respond to an array of biochemical and physical stimuli through cell membrane receptors, signal transduction mechanisms and the synthesis and release of vasoactive, thromboregulatory and growth factor substances. Under basal conditions, factors produced by the endothelium act to maintain vascular homeostasis through the regulation of vascular tone, smooth muscle cell proliferation, inflammation, cellular adhesion and thromboresistance. By maintaining a fine balance of vasodilators, for example NO and prostacyclin, and vasoconstrictors, such as, endothelin and angiotensin II, the endothelium is able to maintain vascular tone.

1.4.1 Nitric Oxide

NO is a key regulator of vascular tone and was first identified as an endothelium-derived relaxing factor following pioneering experiments by Furchgott and Zawadzki in 1980 (Furchgott and Zawadzki, 1980). They discovered that an intact endothelium is required for acetylcholine (ACh) to induce vasodilatation of blood vessels; when the endothelial layer appeared damaged the vessels vasoconstricted. Later studies revealed the vasodilating substance released by the endothelium in response to ACh was NO (Ignarro et al., 1987, Palmer et al., 1987). NO is generated from the amino acid L-arginine by endothelial derived nitric oxide synthase (eNOS), yielding L-citrulline as a by-product (Moncada and Higgs, 1993), and its release is mediated by specific agonists and biomechanical factors like shear stress. There are a number of co-factors also required for NO synthesis, including calmodulin, NADPH, tetrahydrobiopterin (BH_4) and flavin adenine dinucleotide (FAD). NO induces vasodilation by diffusing across the endothelial cell membrane to the vascular smooth muscle cells, where it activates guanylate cyclase (GC) resulting in an elevation of intracellular cyclic guanosine 3', 5-monophosphate (cGMP) concentrations (Figure 1.5) (Moncada and Higgs, 1993).

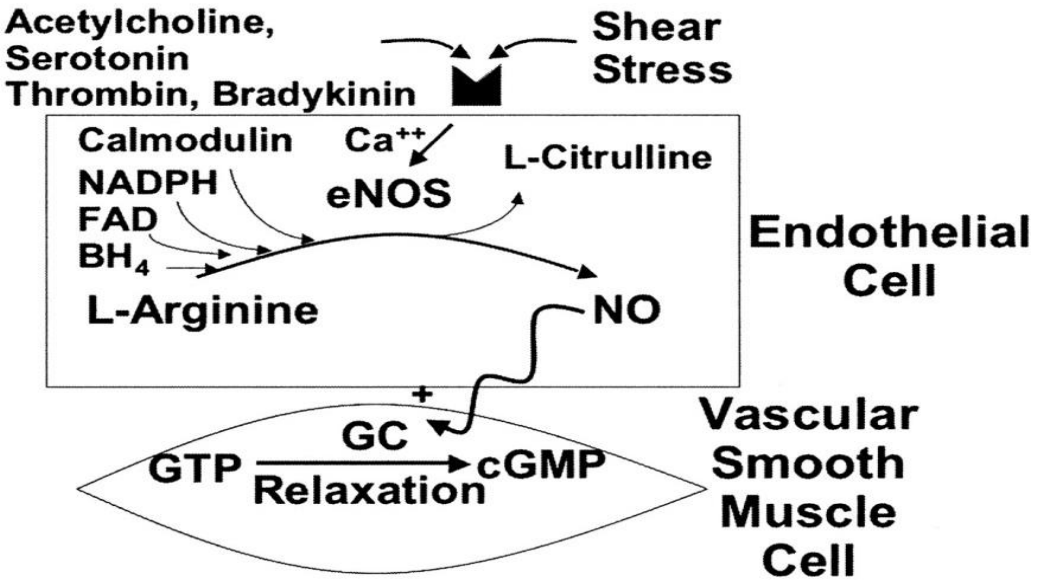


Figure 1.5 The L-arginine: nitric oxide pathway. Different stimuli including shear stress and acetylcholine activate eNOS following the release of calcium from intracellular stores. NO is produced through the conversion of L-Arginine to L-citrulline by eNOS. Various co-factors are needed for eNOS to complete the reaction. NO diffuses from the endothelium to the vascular smooth muscle where it acts on GC resulting in an increase in cGMP and vasodilation. NADPH=nicotinamide adenine dinucleotide phosphate; FAD=flavin adenine dinucleotide; BH4=tetrahydrobiopterin; Ca++=calcium; eNOS=endothelial nitric oxide synthase; NO=nitric oxide; GTP=guanosine triphosphate; GC=guanylate cyclase; cGMP=cyclic guanosine 3', 5-monophosphate. Reproduced with permission of American Society for Nutrition (Gornik and Creager, 2004) via Copyright Clearance Centre.

Although NO is regarded as the major governor of vascular homeostasis it is worth noting the presence of another endothelium derived vasodilator, endothelium derived hyperpolarising factor (EDHF), which has received increased attention over the last few years. The exact nature and mechanism of EDHF is still unknown, however it is recognised as an important contributor to endothelium derived vasodilation. EDHF seems to have the greatest influence in the smaller arteries. Changes in its action are of particular importance for the regulation of organ blood flow, peripheral vascular resistance, and BP, especially when production of NO is compromised (Luksha et al., 2009).

The bioavailability of NO is not only important for the control of vascular tone, its presence is also critical to maintain a quiescent phenotype in the vascular wall through

the inhibition of cellular proliferation, inflammation, cellular adhesion and thrombosis. Therefore NO is a key component in the defence against atherosclerosis.

1.5 The Endothelium and Cardiovascular Disease

In the presence of CV risk factors, for example hypercholesterolemia and hypertension, the endothelium undergoes a phenotypic change. Biochemical and/or biomechanical forces stimulate the endothelium, causing activation, and normal regulatory functions are prevented. The endothelium moves away from a normal, quiescent phenotype and instead begins to mount a host defence response (Deanfield et al., 2007). With this modulation in endothelial phenotype comes numerous challenges, most importantly the bioavailability of NO. With a reduction in NO there is a shift towards vasoconstriction and a pro-atherogenic status. Essential vaso-protective actions are lost leading to disruption of the anti-proliferative state of the endothelium, as well as augmented adhesiveness to blood leukocytes and platelets, increased permeability to plasma proteins and generation of cytokines (Ross, 1999).

Deanfield et al. explained "The fundamental change involved in this process is a switch in signalling from an NO-mediated silencing of cellular processes toward activation by redox signalling" (Deanfield et al., 2007). eNOS is the nitric oxide synthase (NOS) isoform that produces endothelium derived NO and is also capable of producing ROS during endothelial activation. This is referred to as eNOS uncoupling; up-regulation of eNOS in parallel with NADH oxidases leads to the production of peroxynitrite anion ($ONOO^-$) when NO and superoxide combine (Figure 1.6) (Griendling et al., 2000, Harrison, 1997). The uncoupling of eNOS arises when either L-arginine (substrate) or BH_4 (cofactor) are limiting. eNOS, therefore, is at the heart of endothelial homeostasis

due its ability to regulate both silent and activated endothelial phenotypes (Deanfield et al., 2007).

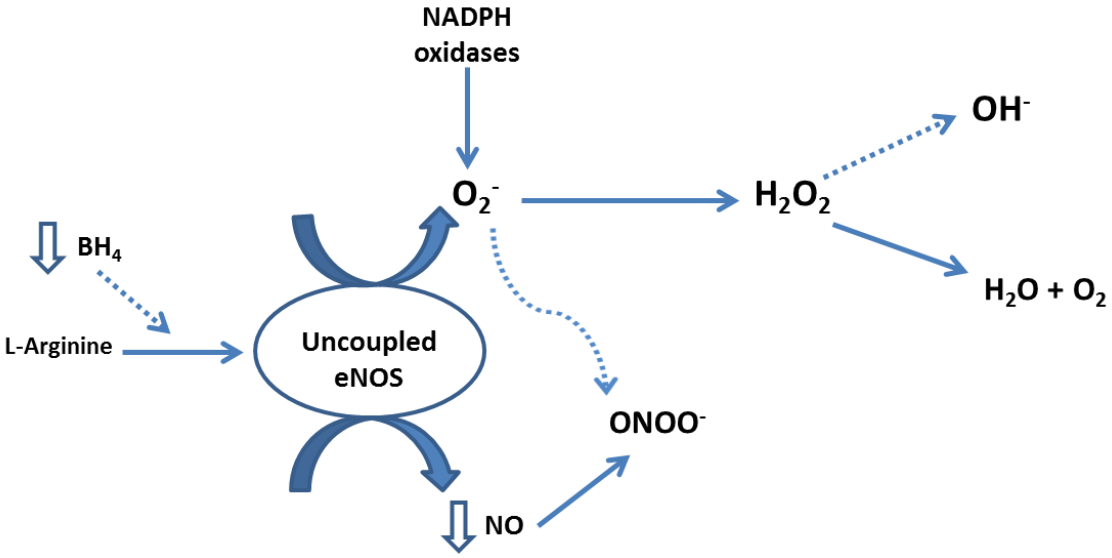


Figure 1.6 eNOS uncoupling. A reduction in BH_4 causes uncoupling of eNOS resulting in the production of O_2^- instead of NO. Peroxynitrite ($ONOO^-$) is formed when NO and O_2^- combine. Superoxide dismutase can scavenge O_2^- resulting in H_2O_2. H_2O_2 can be converted to the OH^- as well as H_2O and O_2. BH_4=tetrahydrobiopterin; eNOS=endothelial nitric oxide synthase; NO=nitric oxide; NADPH=nicotinamide adenine dinucleotide phosphate; O_2^-=superoxide; peroxynitrite=$ONOO^-$; H_2O_2=hydrogen peroxide; OH^-=hydroxyl ion; H_2O=water; O_2=oxygen.

Activation of the endothelium and the subsequent changes in function contribute towards lesion formation and precede the development of atherosclerosis (Luscher and Barton, 1997). Creager et al. 2006 explained that "atherosclerosis is a progressive disease of the medium and large arteries characterised by the accumulation of lipid within the vessel wall that can eventually lead to ischaemia and/or infarction of the heart, brain or extremities". The disease begins during childhood and consists of a long pre-clinical stage before clinical signs start to develop during middle age (Figure 1.7)

Endothelial dysfunction can be reversed or improved by pharmacological and non-pharmacological methods, hence the measurement of endothelial function has the potential to act as a powerful screening tool to address CVD risk and predict future CV events.

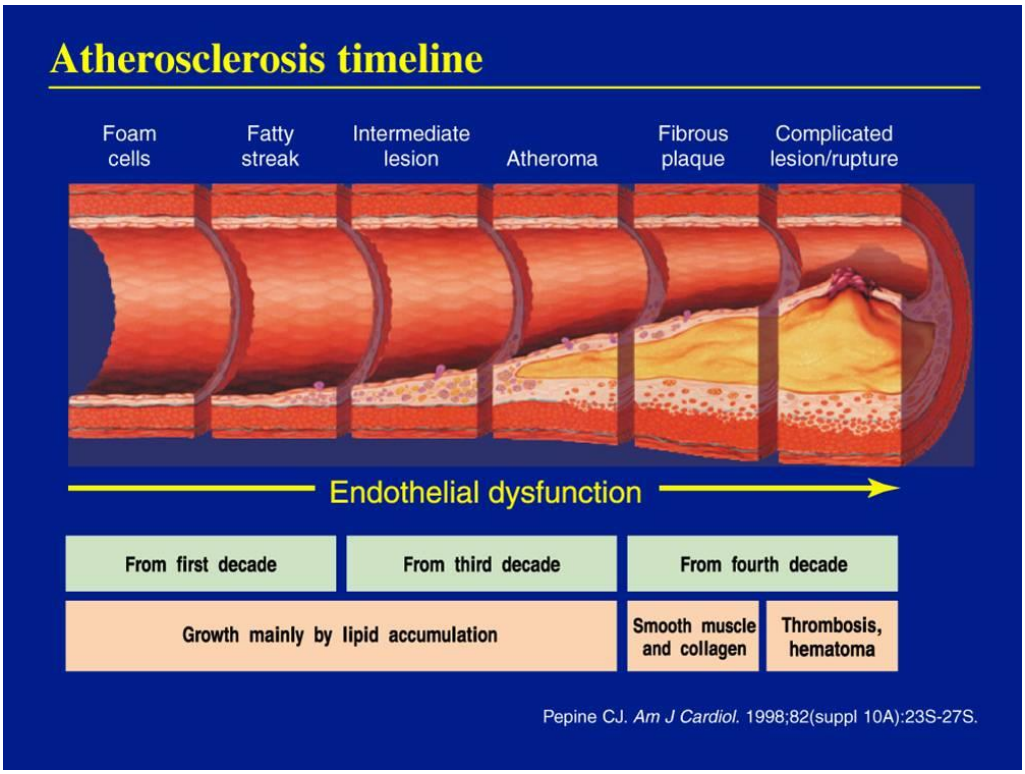


Figure 1.7 The development of atherosclerosis. Changes start to occur in the blood vessel wall from an early age; at around 20 years fatty streaks start to appear in large arteries due to lipid accumulation. With age these fatty streaks progress to lesions and by the fourth decade result in fibrous plaques which can be susceptible to rupture. (Pepine, 1998).

1.6 Assessment of Endothelial Function

The essential role of the endothelium in CVD is recognised by numerous tests currently available to assess its function. At present, a range of tests are often performed to capture a broader understanding of endothelial function as each of the existing methods come with limitations. There still remains a need for the development of a non-invasive test that is cheap, easy to use, reproducible, valid and standardised between laboratories so that it can be used widely in a research setting and also potentially in a clinical setting as a diagnostic/prognostic tool. The test should also be able to detect disease early and predict CV outcome (Flammer et al., 2012). At present none of the techniques used to assess endothelial function meet all of the above essential criteria, as outlined by their limitations in Table 1..

1.6.1 Measures to Assess Endothelial Function

Table 1.1 Advantages and disadvantages of techniques used to assess endothelial function in humans (Deanfield et al., 2007, Widlansky et al., 2003, Leahy, 2007, Flammer et al., 2012).

Method	Advantages	Disadvantages
Coronary Catheterisation	Gold standard invasive test. Assesses endothelial function at the vascular bed of interest. Predicts CV outcome.	Invasive. Expensive. Limited to patients with advanced disease requiring cardiac catheterisation. Repeat testing not possible.
Venous occlusion plethysmography with brachial artery catheterisation	More accessible than coronary arteries. Can assess endothelium during pre-clinical stage. Correlates with coronary arteries.	Invasive. Limited repeatability. Difficult to standardise results. Risk of vascular injury.
High frequency ultrasound with Flow Mediated Dilatation (FMD)	Gold standard non-invasive test. Repeatable. Can be standardised to obtain reproducible results. Predicts future CV events.	Expensive ultrasound machine required. Highly operator dependent. Easily affected by physiological factors.
Peripheral Arterial Tonometry (PAT)	Non-invasive. Easy to use. Repeatable. Reproducible. FDA approved.	Expensive consumables. Digital vascular tone is highly responsive to sympathetic tone.
Laser Doppler Imaging (LDI)	Non-invasive. Repeatable. Reproducible.	Lack of standardisation between different laboratories. May miss rapid changes in blood flow due to the length of time taken to scan.
Full Field Laser Perfusion Imager (FLPI)	Non-invasive. Easy to use. High speed of acquisition. High resolution.	Limited evidence to assess ability to predict CV outcome.
Pulse Wave Analysis (PWA)	Non-invasive. Repeatable. Easy to use. May reflect basal endothelial function.	Influenced by structural aspects of the vasculature beyond the endothelium.
Circulating markers of endothelial function (e.g. ICAM-1 and E-selectin)	Provides important information about severity of endothelial dysfunction.	Can be difficult and expensive to measure.

1.7 Invasive Techniques used to Assess Endothelial Function

The very first clinical studies of endothelial function were undertaken in the coronary circulation using a local infusion of ACh. Changes in vessel diameter were measured by coronary angiography. ACh triggers the release of NO and vasodilatation, when blood vessels contain an intact endothelium, while vasoconstriction develops in individuals with atherosclerotic lesions, indicative of endothelial dysfunction (Figure 1.8) (Ludmer

et al., 1986, Cox et al., 1989).

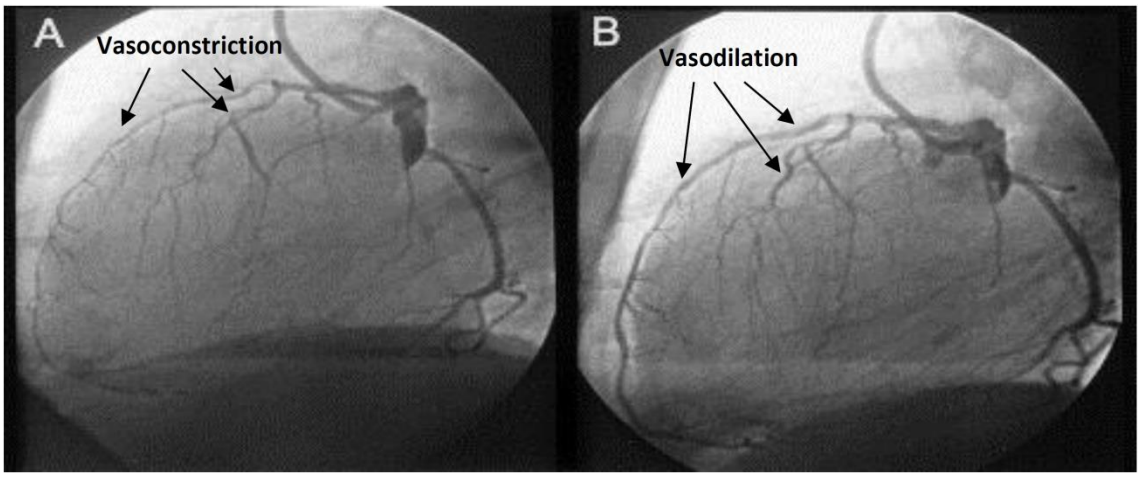


Figure 1.8 Assessment of endothelial function by coronary catheterisation (at 10 days and 9 months post-acute myocardial infarction in the same patient).; in picture A maximal ACh response resulted in vasoconstriction indicating endothelial dysfunction, while in picture B there is vasodilation in response to ACh demonstrating a substantial improvement in the level of endothelial function Adapted from Iraculis et al. (Iraculis et al., 2002).

Development of this technique has allowed for resistance vessel function to be studied through the use of Doppler flow wires. Although recognised as the gold standard invasive test of endothelial function due to directly assessing the coronary arteries, the invasive nature of this technique limits its application to patients with advanced disease and limits repeat testing (Ellins and Halcox, 2011).

Away from the coronary circulation, less invasive studies can be performed more widely in the peripheral circulation, where endothelium-dependent agonists are infused into an artery, normally the brachial artery, and the vasodilator response of the forearm microcirculation can be measured using venous occlusion plethysmography (VOP) (Figure 1.9). This method is based on the measurement of the increase in forearm volume after interruption of the venous return by placing a cuff around the upper arm and inflating to a pressure of 30–40 mmHg. During the occlusion period, the rate of swelling of the forearm can be used to calculate the rate of arterial inflow (Lekakis et al., 2011). The total forearm blood flow measured by VOP is comprised of blood flow

through the skeletal muscle and skin but the technique is unable to distinguish between the two vascular beds. VOP itself is non-invasive, however it is often used in combination with arterial cannulation enabling assessment of the direct effect of drugs on vascular tone. VOP use is still relatively limited, due to its semi-invasive nature if arterial cannulation is used, and difficulties with standardisation between laboratories, for example initial differences in forearm blood flow and arterial pressures, make comparisons between groups and repeat studies problematic (Flammer et al., 2012).

Figure 1.9 Assessment of forearm blood flow using venous occlusion plethysmography at the Vascular and Inflammatory Disease Research Unit, University of Dundee.

1.8 Non-Invasive Techniques used to Assess Endothelial Function

The development of non-invasive techniques has allowed endothelial function to be assessed without the use of needles or surgical intervention in the laboratory by clinical and scientific researchers. However, most importantly, the non-invasive methods have provided the opportunity to investigate the endothelial pathophysiology during the pre-clinical phase of atherosclerosis before clinical symptoms start to develop, at a time when endothelial dysfunction can potentially be reversed.

1.8.1 High Frequency Ultrasound with Flow Mediated Dilatation (FMD)

For non-invasive testing, FMD is the current gold standard for assessing endothelial function. This technique measures vasodilatation in an artery, most commonly the brachial artery, following a period of arterial occlusion in the forearm. A shear stress stimulus is created following a short period of ischaemia, usually 4 to 5 minutes, which stimulates the endothelium to release several vasodilators, including NO, prostaglandins (PGI_2) and EDHF, leading to dilatation of the vessel. The change in diameter of the brachial artery is imaged using high frequency ultrasound (7-12 MHz) and assessed to give an indication of vasomotor function (Figure 1.10) (Corretti et al., 2002).

Figure 1.10 Flow Mediated Dilatation (FMD) patient set up (bottom) at the Vascular and Inflammatory Diseases Research Unit Laboratory, University of Dundee and close up of stereotactic probe holder (top) used to ensure the ultrasound probe is placed directly over the brachial artery throughout the assessment.

A number of outcome studies assessing FMD have shown the technique can identify individuals with increased CVD risk and also predict future CV events. In the Multi-

Ethnic Study of Atherosclerosis (Yeboah et al. 2009) FMD was measured in 3026 subjects free of clinical CVD at baseline. The results from this study showed FMD to be significantly and inversely associated with CV events, such as myocardial infarction or stroke, over a maximum period of 5 years, independent of other key risk factors in a population-based cohort. FMD also provided a substantial improvement in categorising individuals risk when compared to only the FRS; there was a net improvement in the classification of subjects into low, intermediate or high risk categories when compared with the FRS alone. In another study, patients suffering from peripheral arterial disease displayed a reduced hyperaemic response which was associated with increased risk of CVD outcome, for example unstable angina and non-haemorrhagic stroke, over a 2 year follow up. Patients with an event had a lower hyperaemic flow velocity and lower brachial FMD, with FMD proving the strongest predictor of risk in this population (Huang et al., 2007).

More recently the normalisation of FMD to its shear stress stimulus has been studied to improve this measure of endothelial function. Traditionally FMD has only been used to assess changes in brachial artery diameter, but now there is more attention on the FMD hyperaemic velocity component (Figure 1.11), a marker of microvascular function that has been shown to have a stronger relationship with CV risk factors than the actual FMD response itself in some studies.

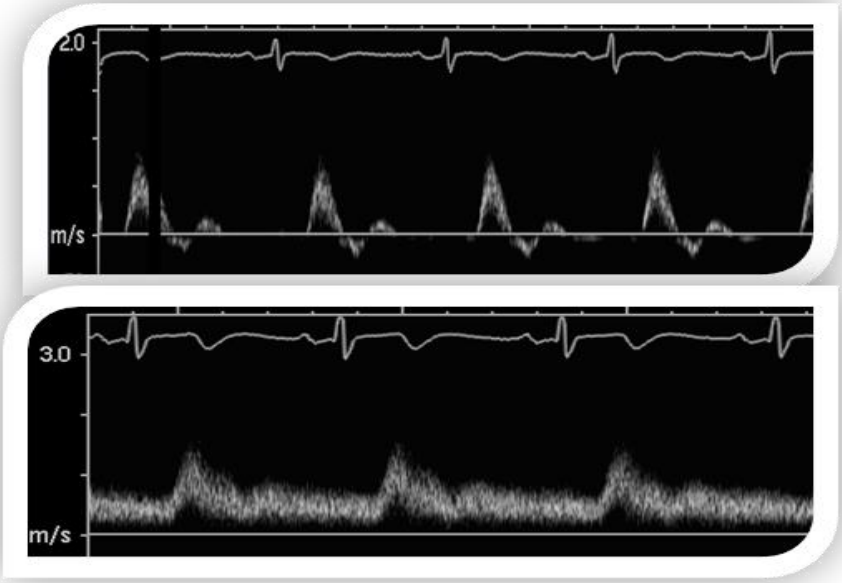


Figure 1.11 Baseline (top) and hyperaemic (bottom) brachial artery velocity traces from the Vascular and Inflammatory Diseases Research Unit Laboratory, University of Dundee.

The Firefighters and Their Endothelium (FATE) study by Anderson et al. 2011 included 1574 male fire fighters free from CVD, who had endothelial function assessed by FMD. Hyperaemic velocity as well as carotid intima medial thickness and CRP were also measured. The hyperaemic velocity, the stimulus for FMD, was found to be associated with adverse CV outcomes in this population, while the FMD response on its own was not. It has been suggested that this alternative assessment of endothelial function may offer better risk stratification for healthy individuals in lower-risk categories and enable better discrimination between moderate and low risk populations (Padilla et al., 2009). Recommendations are to measure both components of this response but the precise method to use for normalisation of shear stress (if at all to do this) has been debated (Parker et al., 2009, Thijssen et al., 2011).

In 2002 a consensus document was released by Corretti et al. providing guidelines for measuring FMD which addressed the strengths and limitations of the technique. Although FMD has strong associations with CVD risk factors and disease, the technique comes with several drawbacks; the technique requires the use of expensive ultrasound

equipment with appropriate software for two-dimensional imaging, colour Doppler, an electrocardiogram (ECG) monitor and a high frequency ultrasound transducer (minimum frequency 7MHz) (Corretti et al., 2002). There is also a need for highly trained operators to perform FMD so a simpler test of reactive hyperaemia using different methodology may in fact have the potential to become a more attractive test of endothelial function. However, the measurement of hyperaemic velocity provides an easier method of endothelial function assessment than standard FMD and has been identified as a significant risk marker for adverse CV outcome. It is therefore worthwhile investigating this novel tool in greater detail and in different sub-populations to confirm these findings.

1.8.2 Peripheral Arterial Tonometry (PAT)

Peripheral arterial tonometry (PAT) is a relatively new, non-invasive technique gaining popularity used to assess endothelial function in the microcirculation (Figure 1.12). Approval from the Federal Drug Administration (FDA) as well as the ease of use has raised the appeal of PAT within leading clinical institutions and research centres. This test measures changes in digital pulse amplitude before and after reactive hyperaemia induced through arterial occlusion of the upper arm (Figure 1.13). Two plethysmograph finger probes are attached to each index finger which monitor changes in finger volume with each arterial pulsation. The probes have a firm exterior with an inflatable chamber inside to impart a uniform pressure on the distal finger. The applied pressure (near diastolic pressure) acts to avert venous pooling and to relieve arterial wall tension (Hamburg and Benjamin, 2009). By placing a finger probe on the contra-lateral index finger without reactive hyperaemia systemic influences can be controlled. In addition to microvascular function PAT technology also provides a measure of arterial stiffness, calculating augmentation index.

Figure 1.12 Peripheral arterial tonometry device: EndoPAT 2000 (Itamar Medical Ltd, Israel).

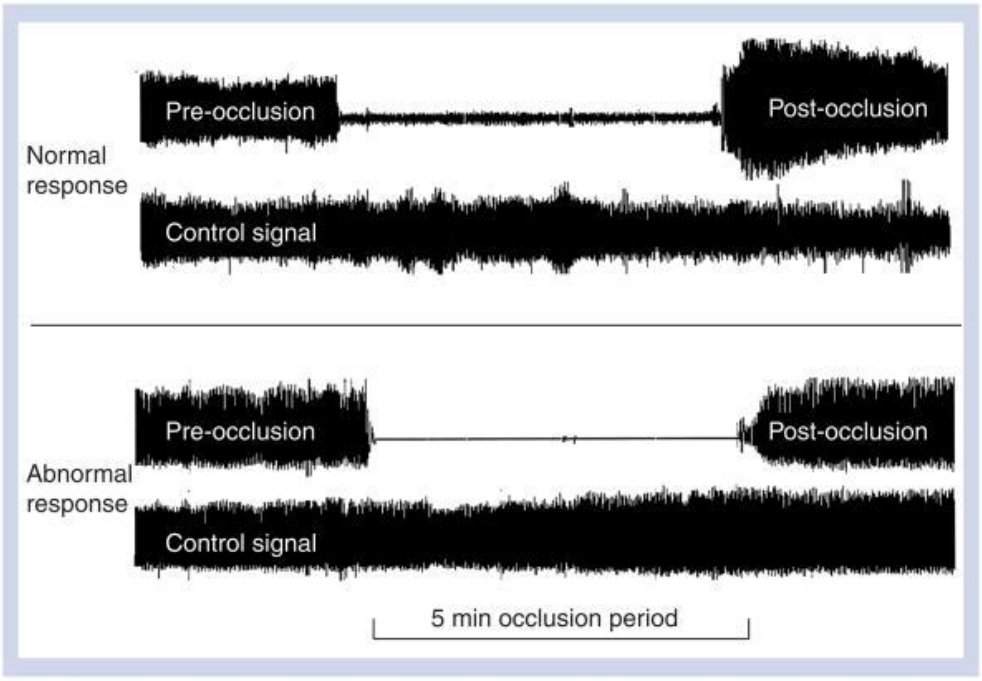


Figure 1.13 EndoPAT traces for a normal reactive hyperaemia response (top) and an abnormal reactive hyperaemia response (bottom) (Reriani et al., 2010). Reproduced with permission of Future Medicine Ltd. via Copyright Clearance Centre.

The main outcome measure calculated by EndoPAT is the reactive hyperaemia index (RHI). The RHI is a ratio of the post occlusion PAT amplitude to pre occlusion PAT amplitude of the study arm divided by the same ratio of the control arm, multiplied by a

baseline correction factor. A threshold of 1.67 or greater is recognised as a good EndoPAT result following the results of a study by Bonetti et al. Bonetti showed EndoPAT correlated with the current gold standard assessment of endothelial function (coronary catheterisation using a local infusion of ACh). The EndoScore can be classified into one of three simple groupings: Red- 1.68 or less, Yellow-1.69-2.0, and Green 2.1-3.0 (Itamar Medical, Israel) indicating the health of the endothelium and the risk of developing future cardiovascular events. A higher EndoScore reflects a healthier endothelium and a lower risk for CVD.

Like FMD, PAT is associated with the prediction of future CVD and subsequent events. In a study by Rubinshtein et al. measurement of endothelial function by PAT predicted late CV events such as myocardial infarction and CV related hospitalisation. A lower RHI, which indicates endothelial dysfunction, was associated with an increased rate of adverse events including myocardial infarction and cardiac death during a 7 year follow up. In this study a natural logarithmic scale RHI (L_RHI) was calculated using the ratio between the digital pulse volume during RH and baseline. A value of 0.4 was used as a cut off to separate low and high RH responses. PAT has also been recognised as an independent predictor of poor prognosis in patients with heart failure and a preserved ejection fraction (Matsue et al., 2012). These studies support the use of PAT as a measure of endothelial function, highlighting the significance and predictive value of digital pulse amplitude. However it must be noted PAT cannot be described as a better predictor of CVD than other tests of endothelial function, including FMD, as currently no single test has a better predictive value over another.

As seen in Table 1., one of the main disadvantages associated with PAT is the cost of the finger probes (approximately €44 per test). Each set of probes can only be used on one occasion, making high consumable costs unavoidable for this test and potentially

preventing the use of this technique in large population studies. Current research has shown that NO plays an important role in PAT and the RHI is reflective of NO availability. Nohria et al. have found that at least half of the increase in digital pulse volume amplitude during reactive hyperaemia (PVA-RH) is dependent on NO, after demonstrating that administration of the NOS inhibitor N^G-nitro-L-arginine methyl ester significantly reduced PVA-RH (Nohria et al., 2006). Although NO contributes to the PAT response, it is only partly responsible meaning other non-endothelial influences are also involved. PAT assesses blood flow in the finger tip, a site which may be affected by both the sympathetic and autonomic nervous systems. Changes in the PVA may in fact be altered by factors such as mental stress and temperature.

1.8.3 Assessment of Skin Microvascular Function

The skin is easily accessible and therefore provides an appropriate site for the assessment of peripheral microvascular function. The skin microcirculation has been used as a circulation model in normal healthy populations as well as various disease states including diabetes, Alzheimer disease, renal disease, and CAD to examine key vascular mechanisms and it is considered to be representative of systemic microvascular function (Turner et al., 2008, Mahe et al., 2012). Skin microvascular function can be assessed using laser Doppler flowmetry/imaging to monitor blood flow in response to a particular stimulus whether it be application of pharmacological agents, thermal challenges (skin heating or cooling) or a mechanical stimulus, such as occlusion of an artery.

Laser Doppler Flowmetry/Imaging

Laser Doppler flowmetry (LDF) is an established non-invasive technique which provides a continuous, sensitive and real-time assessment of blood flow in the skin microcirculation. This technique is based on the Doppler shift of the emitted laser light

when it travels through skin and interacts with static tissue and moving objects, for example red blood cells. The Doppler effect is the relative change in frequency of the light between the moving object and the stationary photo detector. The change in the frequency of the backscattered light is determined by the speed at which red blood cells are travelling (there is no change in the wavelength of the light that is backscattered from static tissue). Following the processing of the resulting photocurrent a blood flow measurement 'flux' is generated which provides information on the average speed of red blood cells and their number concentration. The flux is an estimate of blood flow expressed in arbitrary perfusion units (PU) rather than absolute values since the red blood cell flux is linearly correlated with skin blood flow (Equation 1.1).

$$F = k\bar{s}N \qquad \textbf{1.1}$$

where F = Flux; k = arbitrary constant; $\bar{s}$ = velocity; N = number of red blood cells in the sample.

The first publication demonstrating the use of the Doppler effect to measure blood flow was in 1975, where Stern used a helium-neon laser to assess blood flow in the fingertip under normal conditions and during brachial artery occlusion (Stern, 1975). Since then, LDF has seen numerous developments over the past 30 years and has become a clinically applicable tool by many researchers for the measurement of cutaneous microcirculatory blood flow. LDF offers continuous perfusion monitoring with a single point laser, but cutaneous blood flow is heterogeneous so it is therefore necessary to measure blood flow over a larger region of interest. To accommodate this need laser Doppler imaging (LDI) was developed (Figure 1.14).

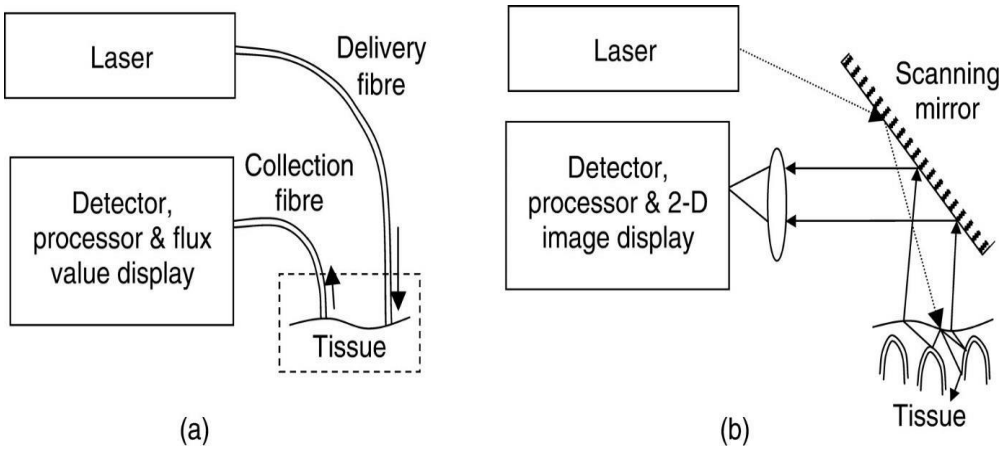


Figure 1.14 (a) Diagram of LDF: single-point probe with an emitting delivery fibre from the source laser light and a collection fibre, which detects and processes the signal; (b) Diagram of LDI. Adapted from Murray et al. (Murray et al., 2004).

LDI works similarly to LDF, measuring skin perfusion based on the Doppler principle, but instead of using the single point method to measure blood flow at a single site LDI scans over a selected area of tissue, reducing spatial variability and thus improving reproducibility. The scattered laser light due to moving red blood cells is analysed and a colour coded perfusion map is produced, allowing skin perfusion to be assessed at a specific area of tissue over time during basal and experimental conditions (Figure 1.15) (Turner et al., 2008). Most LDI systems utilise a helium-neon laser (RED, 632.8 nm) capable of monitoring skin perfusion at a depth of approximately 1.0-1.5mm (Cracowski et al., 2006, Turner et al., 2008). In general, LDI is much slower than LDF due to the scanning action required to measure perfusion over a larger area, making it difficult to assess rapid changes in blood flow (Roustit, 2011). The next sections will describe the most popular techniques used to assess skin microvascular function that are performed using LDF/LDI.

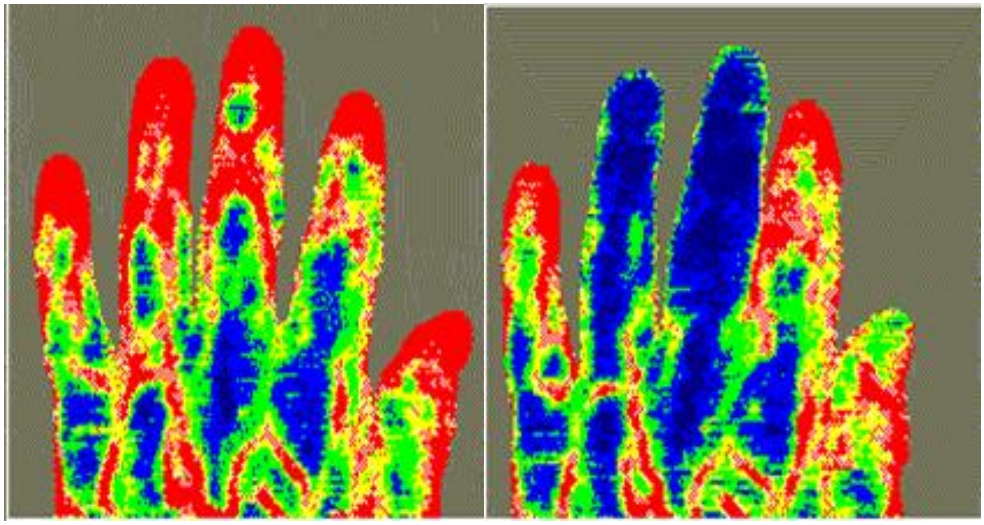


Figure 1.15 Laser Doppler perfusion map of the hand. The different colours in the pictures indicate different levels of perfusion; blue indicates low levels of skin perfusion, green and yellow indicates intermediate levels of skin perfusion and red indicates high levels of skin perfusion. (Moor Instruments, UK).

Iontophoresis

Iontophoresis is a technique used to deliver drugs across the skin non-invasively with the use of a small external electrical current (Kvandal et al., 2003). This method has been used for over 100 years, and was first popularised by Leduc at the start of the 20 century after he successfully introduced strychnine and cyanide ions into rabbits (Leduc, 1908).

Iontophoresis relies on the principal that like charges repel and opposite charges attract each other. Pharmacological drugs that are positively or negatively charged will penetrate through the skin in the presence of an external current based on this rule. For example, ACh, a positively charged drug ion, will migrate across the skin if placed in an ion chamber containing a positively charged electrode (anode) from which it is repelled. The positively charged drug ion is then attracted to the negative electrode (cathode) at another site on the skin, which is needed to complete the circuit (Figure 1.16).

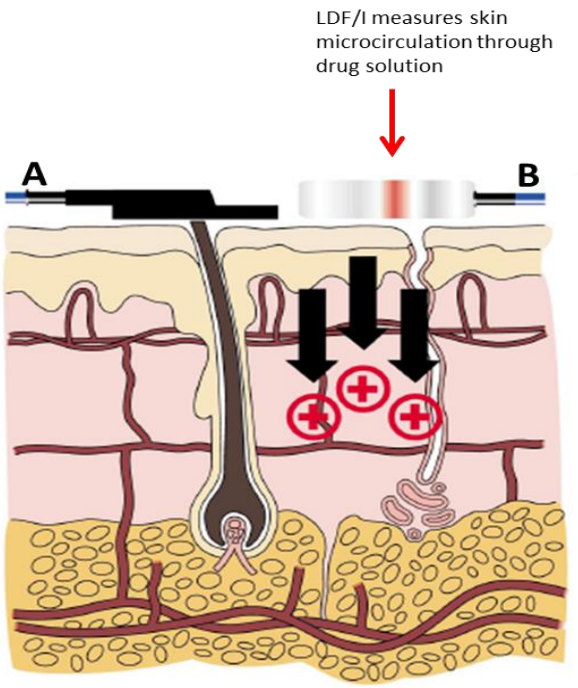


Figure 1.16 The iontophoresis technique. A: Negatively charged reference electrode; B: Ion chamber containing positively charged drug ions. The electric field at the ion chamber (B) repels the drug ions which are forced through the skin surface. Adapted from Moor Instruments, UK.

The main advantage of iontophoresis is the ability to deliver vasoactive drugs across the skin non-invasively without the use of any needles. The small quantity of drug delivered also means that there is minimal risk of over-dosing. There are several limitations to iontophoresis; importantly the exact quantity of drug delivered is unable to be calculated precisely, affecting the accuracy of perfusion results. In response to the delivery of the drugs using the iontophoretic system, minor side effects can occur in patients such as reddening, itching and irritation of the skin surface. The major drawback associated with LDF/LDI and iontophoresis is the lack of standardisation of protocols between different laboratories (Roustit, 2011, Turner et al., 2008). This makes it difficult to make comparisons between different studies. If a specific protocol was developed and agreed this would help to resolve the differences currently seen in blood flow results between sites.

LDF/LDI together with iontophoresis have been used extensively to measure endothelial function before, during and after stimulation in normal populations, as well as populations with pathological conditions, including type 1 diabetes. Khan et al. found young type 1 diabetes patients had a decreased response to ACh and sodium nitroprusside (SNP) compared to control subjects (Figure 1.17), and this blunted response was linked to duration of diabetes and level of glycaemic control (Khan, 2000). In another study by Elhadd et al. a decreased response to ACh was also observed in young adult type 1 diabetes patients, compared with a group of prepubertal type 1 diabetes patients (Elhadd et al., 1990). An impaired response to iontophoresis demonstrated by a reduced response to ACh and SNP is indicative of poor vasodilator and endothelial function.

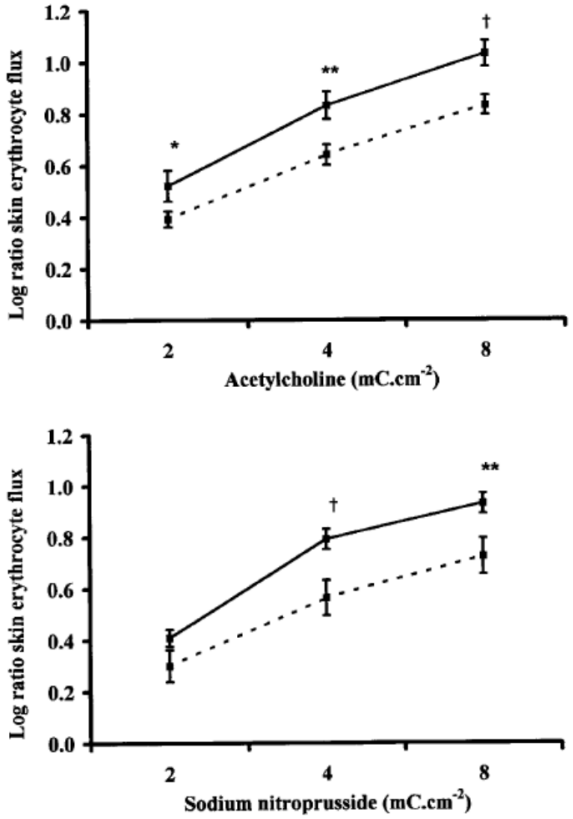


Figure 1.17 Patients with type 1 diabetes display a significantly lower response to iontophoresis of acetylcholine (top) and sodium nitroprusside (bottom) compared to control subjects. Results expressed as logarithmic ratios (response over baseline). Type 1 diabetes patients [- - - - -] and control subjects (n = 25) [——]. *P 0.05,**P 0.01,†P 0.005 (post-hoc t tests).

Reduced cutaneous ACh-induced vasodilatation has also been observed in patients known to have an increased risk of CVD (Ijzerman et al., 2003). Furthermore, vasodilation in response to ACh in the skin has been shown to correlate with coronary microvascular function (Khan et al., 2008) highlighting the importance of this technique in identifying patients at risk of developing CVD or individuals who are showing early signs of disease (Ijzerman et al., 2003).

Post Occlusive Reactive Hyperaemia

Post occlusive reactive hyperaemia (PORH) represents the increase in microvascular blood flow following a short period of arterial occlusion. The increase in blood flow causes an increase in shear stress against the vessel wall, which results in the production of several vasodilators, including NO, adenosine, and $PGI_{2,}$ leading to vasodilation of the microcirculation.

PORH is a method used to measure microvascular reactivity in the skin. The test involves placing a sphygmomanometer cuff around the limb of choice; in most cases the arm (forearm/upper), but the calf or thigh may also be used. The cuff is inflated to a suprasystolic pressure for a designated time, usually between 3 and 5 minutes, and the pressure is released rapidly at the end of the occlusion period, resulting in an increase in blood flow above baseline levels. Skin perfusion is monitored throughout the assessment providing a blood flow measurement before, during and after occlusion, ensuring the maximum increase in perfusion is observed (Figure 1.18).

The main parameter derived from the test is the peak response either in absolute values or expressed as a function of baseline. The AUC can also be calculated taking into account the time taken for skin perfusion to return to baseline levels. The PORH response has been shown to be blunted in patients with CV risk, and a recent

publication found PORH to be an independent marker of atherosclerotic damage in patients with type 1 diabetes (Strain et al., 2010, Rossi et al., 2011, Rossi et al., 2013).

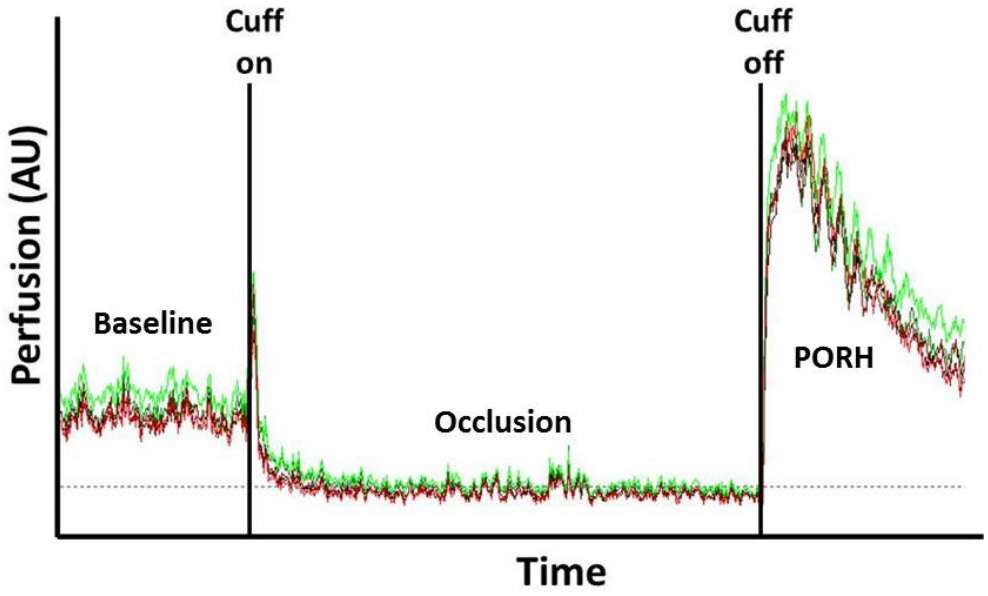


Figure 1.18 A typical post occlusive reactive hyperaemia trace of skin perfusion. The three phases of the test are shown; baseline, occlusion (when the cuff is inflated) and hyperaemia (immediately after cuff release).

A normal PORH response is elicited by a quick increase in blood flow above baseline levels immediately after cuff release, followed by a gradual decrease in blood flow, before returning to resting levels. The exact mechanisms of reactive hyperaemia are still unknown but they are believed to involve contributions from the following: release of vasodilator mediators and metabolites from ischaemic tissue, myogenic mediated relaxation of blood vessels and sensory nerves (Patterson, 1956, Kontos et al., 1965, Minson and Lorenzo, 2007). Numerous local mediators are produced in the hypoxic tissues during the occlusion phase of the test which can act on the surrounding smooth muscle cells and stimulate vasodilation of blood vessels (Kontos et al., 1965). The myogenic component of the reactive hyperaemia response occurs due to a reduction in arteriole intravascular pressure as a result of arterial occlusion (Lombard and Duling, 1977). In addition, sensory nerves have more recently been shown to play an important

role in vasodilation after it was discovered that inhibiting sensory nerves decreased the reactive hyperaemia response in the skin (Larkin and Williams, 1993, Minson and Lorenzo, 2007).

In the literature there are conflicting views on the contribution of NO to reactive hyperaemia. Some studies have shown that only immediately after a period of arterial occlusion is the vasodilator response most dependent on NO (Raff et al., 2010). Studies have also identified nitric oxide to be important in the maintenance of vasodilation after the initial peak phase (Tagawa et al., 1994) and others have shown NO contributes throughout the reactive hyperaemia response (Dakak et al., 1998).

Local Heating

Local skin heating can be performed to measure the maximum vasodilator capacity of the skin. By heating the skin, blood vessels dilate and the increase in blood flow can be measured using LDF/I. An increase in blood flow can also be achieved through capillary recruitment, where the number of perfused capillaries increases in response to a stimulus, in this case skin heating. Local skin heating between 42^0C and 44^0C induces a biphasic increase in blood flow. An initial peak is evident in the first few minutes, which is predominantly axon reflex mediated, followed by a brief dip and then a sustained plateau which depends on endothelial factors, largely NO (Figure 1.19) (Roustit, 2011). This test offers the ability to monitor both axon vasodilation and endothelium-dependent vasodilation.

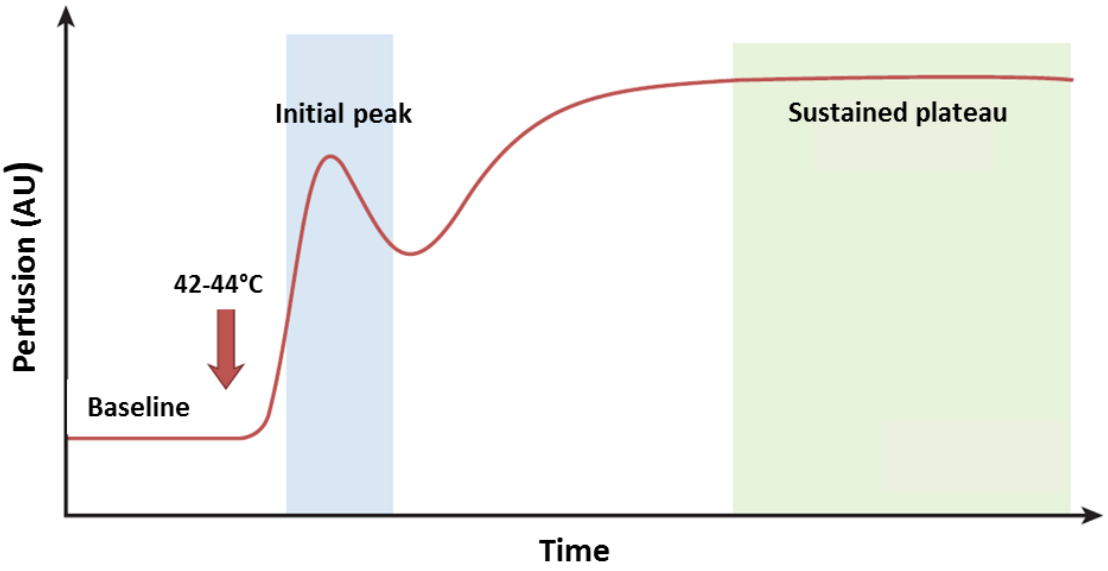


Figure 1.19 A typical local heating response showing the biphasic increase in blood flow. On skin heating there is an initial increase in skin perfusion, followed by brief dip before a sustained plateau. Adapted from Roustit and Cracowski (Roustit and Cracowski, 2013).

The local heating test requires a specialised probe which allows a constant temperature to be maintained throughout the assessment. Heating protocols typically last approximately 30 minutes so that the plateau phase can be observed. If the test is longer than 50 minutes blood flow will start to slowly decrease towards baseline (Roustit and Cracowski, 2012).

In research, LDF together with local heating has revealed a reduced cutaneous endothelial vasodilator response in essential hypertensive patients (Smith et al., 2011). In addition, similar results were also found in patients with type 2 diabetes (Beer et al., 2008, Smith et al., 2011).

1.8.4 Full Field Laser Perfusion Imager

The work in this project will assess the full field laser perfusion imager (FLPI) as a new potential device for assessing endothelial function (Figure 1.20). FLPI measures blood flow within the microcirculation by laser speckle contrast imaging (LSCI), a technique first used to study the retinal vasculature by Fercher and Briers in the early 1980's.

FLPI uses a near infra-red laser diode (785 nm), which captures images from the superficial microvessels at a depth of around 300 μm. Similarly to LDI, LSCI illuminates the skin, and moving red blood cells within the tissue cause this light to be backscattered.

Figure 1.20 Full Field Laser Perfusion Imager (FLPI) (Moor Instruments, UK).

In the case of LSCI, the back scattered light is focussed through a limiting aperture resulting in diffraction, and this diffracted pattern, referred to as speckles, is captured by a camera (Senarathna et al., 2013). The speckle pattern is altered by moving red blood

cells within a specific region of interest; when there is a high flow rate the speckle pattern appears blurred and the contrast decreases whereas a high contrast indicates low flow (Briers, 2001). The contrast image generated is processed and converted into a colour coded image, representing the blood flow within a particular region of tissue. This device can be used to assess blood flow responses to a range of stimuli as mentioned in section 1.8.3, but this project will focus specifically on the PORH test.

One major difference between FLPI and LDI is the depth at which the laser can penetrate the skin. FLPI measures to a depth of approximately 300 μm, so therefore assesses superficial cutaneous perfusion. The LDI however, has a depth of approximately 1.0-1.5 mm so is able to penetrate slightly deeper. The novelty of FLPI over LDI is the speed of acquisition. FLPI is capable of capturing real time images (at 25 images per second), up to four times faster than LDI (Leahy, 2007). Previously dynamic changes could only be measured using a single point laser LDF. LDF can measure continuously but over a very small area, unlike FLPI which offers scans over a large region of interest (from 5mm x7mm to 15 x 20 cm). The small area scanned by LDF ($1mm^3$), can often result in poor reproducibility. For the purpose of measuring PORH for example, FLPI is ideally suited to capture dynamic changes in blood flow over a large area of tissue, which would be missed by conventional LDI scanners. A limitation of FLPI and the LSCI technique is its sensitivity to movement artefacts, which is more of a problem than standard laser Doppler techniques. Despite this, the novel technology provides an imaging system with advantages over both the LDF and LDI techniques and therefore has the potential to become an important tool in clinical research.

It is interesting to note that the beginnings of endothelial dysfunction may in fact begin within the microcirculation, before developing in the large conduit arteries (Isaksson et

al., 1993). Therefore FLPI, which can assess endothelial function in the microvessels using PORH, may be able to identify early signs of disease before detection can be made in larger vessels.

FLPI has further advantages over existing non-invasive methods of endothelial function. The current gold standard non-invasive test used to assess endothelial function, as mentioned in section 1.8.1, is FMD. However, unlike FMD, FLPI does not require highly trained operators, instead providing a much simpler test for reactive hyperaemia with minimal operator training. PAT is another non-invasive test being widely used to assess endothelial status, and compared with FMD provides a more straight forward means of investigation. However the cost of consumables needed to perform this test may make this test less appealing to researchers. With just a single one off payment, FLPI in combination with PORH offers a test of endothelial function with considerably greater value for money compared with PAT. FLPI coupled with PORH could become a useful test for the measurement of endothelial function, with advantages over the already established methods of LDI, FMD and PAT.

1.8.5 Pulse Wave Analysis

Pulse wave analysis (PWA) is most commonly thought of as a method of assessing arterial stiffness and wave reflection, providing valuable information about the mechanical properties of the arterial system, but this simple, non-invasive measurement can also be used to assess endothelial function (Figure 1.21) (Stoner et al., 2012). Using PWA, endothelial function can be investigated by monitoring the effects of salbutamol (a beta 2 agonist) on augmentation index (AIx), providing an assessment of systemic arterial stiffness. PWA has many advantages as outlined in Table 1., but at present additional clinical studies are needed to further validate this technique.

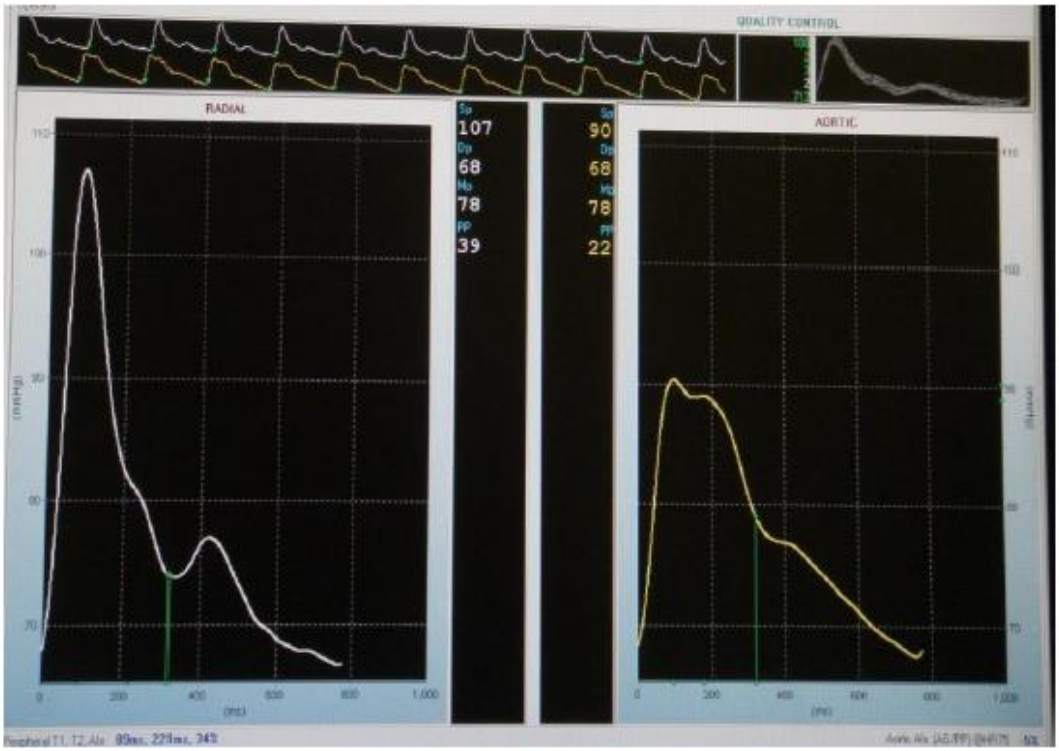


Figure 1.21 A typical pulse wave analysis trace (top) from the Vascular and Inflammatory Diseases Research Unit, University of Dundee and the SphygmoCor device used to capture the measurement (bottom) (ScanMed Medical Supplies, UK).

1.9 Circulating Markers of Endothelial Function

An additional method to non-invasive testing which looks at systemic endothelial function is the measurement of the products of endothelial cells and markers of endothelial damage and repair found in circulating blood. Upon endothelial activation there are changes to inflammatory cytokines, adhesion molecules, regulators of thrombosis and NO biology, which provides useful information about the extent of endothelial dysfunction in specific patient groups and adds to physiological assessments of endothelial function. These circulating markers have the potential to provide an assessment of patients CV status and severity of endothelial damage, however it is both costly and difficult to measure these markers meaning, at present, application of this method is confined to clinical research. It is not within the scope of this review to discuss these markers in detail, but a list of the main biomarkers of endothelial function are shown in Table 1.2 below.

Table 1.2 Circulating markers used to assess endothelial function.

Vascular Markers of Endothelial Function	References
Intercellular Adhesion Molecule-1	(Dessein et al., 2005, Barone Gibbs et al., 2012)
Vascular Cell Adhesion Molecule-1	(Dessein et al., 2005, Barone Gibbs et al., 2012)
E-selectin	(Kistorp et al., 2008, Dessein et al., 2005, Barone Gibbs et al., 2012)
P-selectin	(Barone Gibbs et al., 2012)
Endothelin	(Saini et al., 2011)
von Willebrand factor	(Kistorp et al., 2008)
Tissue Plasminogen Activator	(Barone Gibbs et al., 2012)
Endothelial Progenitor Cells	(Fadini et al., 2012, Bruyndonckx et al., 2013)
Endothelial Microparticles	(Bruyndonckx et al., 2013)

1.10 Summary of the Clinical Implications of Endothelial Dysfunction

There is substantial evidence in the literature to show that endothelial dysfunction in both the macrovasculature and microvasculature is associated with CV risk factors and disease. Endothelial dysfunction has been detected in patients with hypertension, diabetes, dyslipidaemia, inflammatory diseases, as well as in smokers and obese individuals (Flammer et al., 2012). Damage to the endothelium results as a consequence of these risk factors, therefore the endothelium is regarded as a sensor of total CVD risk and vascular health.

The endothelium has proven to be a significant predictor of future CV events since the very first assessments were performed in the coronary vasculature in patients with and without CAD. Despite this, for the purpose of primary prevention, it is not possible to perform invasive assessments and instead many studies focus on peripheral endothelial function, which has been shown to correlate with coronary endothelial function, and predict CV events beyond CV risk factors. Endothelial function measured by FMD has successfully predicted future CV events following adjustment for traditional risk factors (Yeboah et al., 2007) and the FRS (Yeboah et al., 2009) in a primary prevention setting. In addition, FMD and the FRS together provided a better model for classifying CV risk compared to FMD or FRS separately (Yeboah et al., 2009), evidence that endothelial function does add to information learned from simply looking at CV risk factors. Studies have also shown microvascular endothelial function to be associated with CV events in older patients (Lind et al., 2011) and to be predictive of non-obstructive coronary atherosclerosis (Matsuzawa et al., 2010) which is poorly predicted by the FRS. By combining microvascular endothelial function with the FRS better risk discrimination can be made.

Through the assessment of macrovascular and microvascular endothelial function a better understanding and classification of CV risk can be determined in comparison with the use of traditional CV risk factors alone. Microvascular dysfunction may precede macrovascular dysfunction, suggesting that microvascular endothelial function tests may be more valuable in younger subjects free from disease, while macrovascular assessments may be better suited in patients with existing CVD.

Although endothelial function has been shown to be predictive of future CV events, the measure has not yet been adopted into the recommended guidelines by the American Heart Association/American College of Cardiology or the European Society of Cardiology (Flammer et al., 2012). The omission from these guidelines may be related to the shortage of clear prognostic value from endothelial function assessments and a lack of standardisation of some non-invasive techniques with the exception of PAT. In addition, most of the data demonstrating the predictive qualities of FMD was published after these recommended guidelines were released (Flammer et al., 2012). However other methods used to assess vascular function such as carotid ultrasound and ankle brachial pressure index (ABI) have been included in the aforementioned guidelines under class IIa (level B). This grouping suggests it is "reasonable to perform these procedures" and there is "recommendation in favour of procedure being useful" (Greenland et al., 2010). In addition to carotid ultrasound and ABI, carotid-femoral pulse wave velocity is also included in the European Society of Hypertension guidelines under the same class of recommendation as the American Heart Association and American College of Cardiology. These guidelines state there is a "weight of evidence in favour of usefulness" for these methods (Mancia et al., 2013). Future guidelines may include recommendations for the assessment of endothelial function to complement the other vascular tests included at present.

Not only has endothelial function been shown to be predictive in primary prevention, but it has also been predictive in secondary prevention in patients with established CVD. The predictive qualities of endothelial function in secondary prevention were first seen in patients with non-obstructive coronary artery disease, who had a greater incidence of CV (Schachinger et al., 2000, Suwaidi et al., 2000) and cerebrovascular events with reduced coronary vascular function (Targonski et al., 2003) . In established CAD, increased rates of CV events were found in patients who displayed endothelial dysfunction, compared to patients with normal endothelial function (Lerman and Zeiher, 2005). Endothelial dysfunction therefore is important in secondary prevention, due to its involvement in the progression and development of atherosclerosis.

An improvement in endothelial function has been used as a marker of successful treatment in a wide range of intervention studies including pharmacological agents, dietary modifications and nutritional supplements. The cholesterol lowering drug statins was the first agent used to demonstrate positive effects on endothelial function almost 20 years ago (Anderson et al., 1995, Treasure et al., 1995). Since then improvements in coronary and peripheral endothelial function have been well documented in the literature, thought to be linked to the anti-oxidant and anti-inflammatory properties of the drug and its ability to restore NO bioavailability (Bonetti et al., 2003). Anti-hypertensive agents have also been shown to improve endothelial function and even reverse endothelial damage, with the ACE inhibitors proving especially important (Taddei et al., 1998b). Through vasodilation of blood vessels, by decreasing the amount of calcium entry through L-type voltage dependent channels in the membrane of smooth muscle cells and improving NO bioavailability, calcium channel blockers can also enhance endothelial function (Tang and Vanhoutte, 2010).

Dietary modifications, particularly the addition of foods rich in polyphenols such as cocoa, fruits and tea, have beneficial effects on endothelial function. Cocoa supplementation has been shown to improve FMD in healthy subjects, patients with at least 1 CVD risk factor, smokers, hypertensive patients and patients with diabetes (Heiss et al., 2003, Hermann et al., 2006, Grassi et al., 2008, Balzer et al., 2008). Furthermore, fish oil supplementation, in the form of long-chain n-3 poly unsaturated fatty acids, has also been shown to improve endothelium-dependent vasodilation in healthy subjects, assessed by iontophoresis of ACh (Khan et al., 2003).

Treatments shown to improve CVD disease risk and mortality rates concordantly enhance endothelial function, but it is not clear if the reverse is also the case. For instance intake of vitamin C, vitamin E and folic acid supplements is associated with an improvement in endothelial function acutely (Taddei et al., 1998a), but this benefit is yet to be seen over a longer period of time or in CVD prevention (Kinlay et al., 2004).

The effects of lifestyle changes on endothelial function have been investigated. Both physical exercise and weight reduction have been shown to improve endothelial function as a result of increasing the bioavailability of NO. Smoking cessation has a beneficial effect on endothelial function, specifically coronary endothelial function (Hosokawa et al., 2008).

At present, assessment of endothelial function is not currently recommended for clinical use and it is predominantly performed in a research setting. It is difficult to measure endothelial function clinically due to its heterogeneous functions and also because there is not a single test capable of capturing a full physiological profile of the complete vascular tree. The ability to measure endothelial function non-invasively in the peripheral circulation offers a valuable tool which can identify risk factors, explain mechanisms of vascular/endothelial dysfunction and be used as a surrogate marker of

CV risk in intervention studies investigating novel treatments. Further research is required to study the clinical utility of endothelial function testing to assess CV risk and therapeutic intervention.

Endothelial dysfunction is the earliest detectable functional indicator of CVD and contributes towards lesion formation and the development of atherosclerosis. It is not surprising therefore that a wide variety of tests are currently available to assess endothelial function, however there still remains the need for a new test which will be able to meet all the desired aforementioned criteria and become acceptable for clinical application for risk stratification. The existing tests all come with their own limitations which make it difficult to identify a single superior test despite the presence of gold standard invasive and non-invasive methods. As a result an array of assessments will usually be selected to provide a thorough investigation of endothelial health. This project explores the possibility of developing a new method of measuring endothelial function that could potentially provide a clinically applicable assessment. The statistics of CV mortality emphasise the importance and urgency of finding a new test of endothelial function which is capable of identifying CVD in its earliest form to give the greatest opportunity for improvement, or even reversal, of endothelial damage.

1.11 Aims/Objectives

1. To investigate whether skin microvascular function is a good marker of overall vascular function when compared with the gold standard non-invasive assessment, FMD.
2. To develop a test of microvascular endothelial function using PORH, measured by FLPI, that provides optimal reproducibility based on cuff location, measurement site and skin temperature.

3. To compare this developed test of PORH measured using FLPI with the EndoPAT device, the only FDA validated measure of endothelial function, in two groups of normal healthy volunteers free of symptomatic CVD (Group 1 *n*=15: 18-30 years; Group 2 *n*=15: 40-70 years).
4. To investigate the relationship between forearm skin perfusion and brachial artery velocity, the stimulus for FMD, at baseline and during hyperaemia.

1.12 Hypotheses

1. The developed test of PORH measured using FLPI (and PAT) will be able to detect age related changes in endothelial function between Group 1 and Group 2.
2. There will be a relationship between forearm skin perfusion, measured using FLPI, and brachial artery velocity, two methods used to assess microvascular function.

Chapter 2

Skin Microvascular Function: A Good Marker of Overall Vascular Function?

This chapter will explore the relationship between skin microvascular function, assessed using laser Doppler, and FMD, the current gold standard non-invasive technique used to measure endothelial function, to determine if the skin microcirculation is representative of general vascular function (within the macrocirculation). In particular, given that the signal from LDI consists largely of a velocity component, the relationship between the skin microvasculature and the velocity component of FMD, from the Doppler spectrum, will be investigated.

In recent years, the normalisation of FMD to its shear stress stimulus (brachial artery velocity) has been investigated and has since been recognised as an important marker of microvascular function (Anderson et al., 2011, Huang et al., 2007). The brachial artery velocity relates to the resistance vessels downstream of the brachial artery in the forearm, a site where LDI in combination with iontophoresis can be performed to assess the skin microcirculation. Research studies have shown the brachial artery velocity to be associated with adverse CV outcomes (Huang et al., 2007, Anderson et al., 2011). Furthermore, evidence suggests that this parameter may provide a better method for risk stratification than the traditional FMD response, especially in healthy subjects who are at low risk (Padilla et al., 2009).

2.1 Methods

2.1.1 Study Participants

A database containing data from previous research studies within the Vascular and Inflammatory Diseases Research Unit, University of Dundee from 2000 to 2012 was used for this part of the project. The database was comprised of healthy subjects free from CVD and patients with cardiac syndrome X, rheumatoid arthritis, peripheral arterial disease or chronic fatigue syndrome.

2.1.2 Study Procedures

Subjects were instructed to fast and refrain from caffeine, alcohol and tobacco for at least 2 hours before testing. Subjects were positioned comfortably in a supine position in a temperature controlled room (23-25ºC) and underwent an acclimatisation period of 10 minutes before testing began.

2.1.3 Assessment of the Skin Microcirculation using Laser Doppler Imaging with Iontophoresis

Forearm skin microvascular function was assessed by measuring the response to iontophoresis of ACh (Miochol-E, Novartis, Surrey, UK), an endothelium-dependent vasodilator, and SNP (Rottapharm, Barcelona, Spain), an endothelium-independent vasodilator. Prior to flux recordings, the volar surface of the right forearm was prepared for measurements. A piece of adhesive tape was used to remove any dead skin cells on the surface of the skin and an alcohol swab was used to wipe clean the area of skin before it was gently dried with a tissue. The iontophoresis chambers (Moor Instruments Ltd, Axminster, Devon, UK) used to hold the drugs had a 20mm internal diameter, with a wire electrode around the inner surface and were fixed to the skin using a double sided adhesive ring. ACh and SNP were dissolved in de-ionised water to a 1 % solution and a 2ml solution was added to the appropriate iontophoresis chambers (Figure 2.1). The

drugs were delivered at the same time using consecutive increases in anodal (ACh) and cathodal (SNP) currents. Changes in skin perfusion were measured using LDI (moorLDI, Moor Instruments Ltd. Axminster, Devon, UK). A colour coded perfusion map is generated based on laser Doppler flux, which is proportional to the speed and number concentration of red blood cells, providing a representation of skin blood flow in PU (Figure 2.1). Following two baseline scans, ACh and SNP were delivered using an incremental current of 10, 15, 20, 50 and 100μA. The median value of each individual scan is recorded and then the mean of the two highest stable scan values for each dose is calculated using analysis software (moorLDI software version 5.3, Moor Instruments Ltd, Axminster, Devon, UK). The AUC was calculated for ACh and SNP using the following equation:

$$AUC(units) = (Mean\ of\ all\ scans\ \times time) - (Mean\ Baseline\ \times time) \quad \textbf{2.1}$$

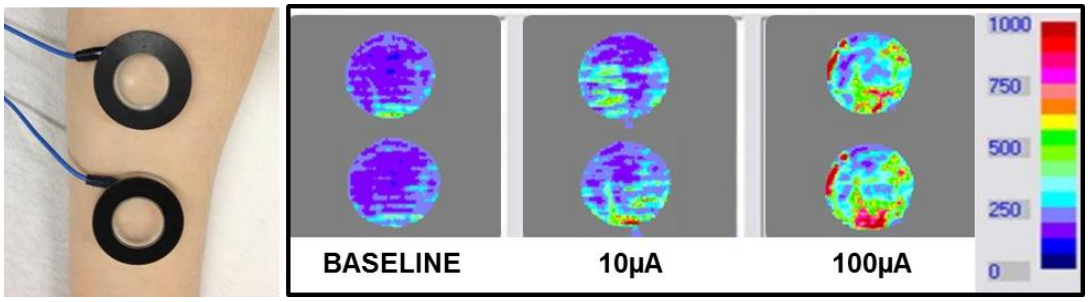


Figure 2.1 The iontophoresis chambers used to deliver ACh and SNP (left) and a colour coded perfusion map displaying increased skin perfusion following iontophoresis of ACh (top) and SNP (bottom) at increasing currents (10 and 100μA).

2.1.4 Assessment of Flow Mediated Dilatation and Brachial Artery Velocity

FMD of the brachial artery was performed according to standard guidelines (Corretti et al., 2002) on the same day as LDI and iontophoresis. Using an Acuson Sequoia C512 ultrasound system (Siemens Medical Solutions USA Inc., Malvern, USA), the brachial artery was imaged in longitudinal section with a 5 to 8 MHz linear array transducer (Figure 2.2). An adjustable snake-arm clamp was used to hold the transducer in position to ensure the same section of the artery was imaged throughout the study (Figure 2.2).

Images were taken at the end of diastole, in order to prevent variations in vessel diameter caused by the arterial pulse, using the R-wave trigger of an ECG trace. Baseline images of the brachial artery were recorded for 1 minute before a blood pressure cuff, positioned around the forearm, was inflated suprasystolically (minimum 200 mmHg). After 5 minutes, the cuff was released and images were collected for 2 minutes. In addition to brachial artery diameter, pulse-wave spectral Doppler recordings were obtained pre-occlusion and on cuff deflation at the brachial artery (Figure 2.3); baseline velocity was considered to be the average of three Doppler tracings before occlusion began, and hyperaemic velocity was considered the average of three Doppler tracings after cuff release. The brachial artery images and velocities were analysed using Vascular Tools Brachial Analyser and Doppler Flow Analyser for Research (Medical Imaging Applications LLC, Coralville, USA) to determine FMD, the flow velocity integral (AUC) and the maximum flow velocity. FMD was calculated as the maximum percentage change in diameter following cuff release relative to baseline diameter.

Figure 2.2 FMD patient set up (top) and close up of the transducer positioned above the antecubital fossa (bottom), Vascular and Inflammatory Diseases Research Unit.

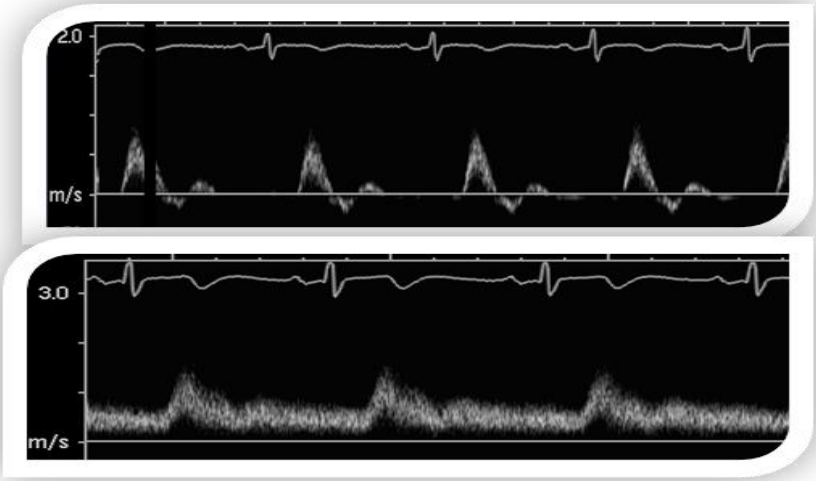


Figure 2.3 An example trace of baseline (top) and hyperaemic (bottom) brachial artery velocities, Vascular and Inflammatory Diseases Research Unit.

2.1.5 Statistical Analysis

A Pearson correlation was selected for statistical analyses. For all statistical tests a *p* value of <0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS 18 (SPSS Inc., Illinois, Chicago, USA).

2.2 Results

The assessment of skin microvascular function using iontophoresis of ACh and SNP at the forearm was investigated to see how it related to the microvascular resistance response for FMD by examining the brachial integral and maximum velocities, which provides the stimulus for FMD.

The demographics and key vascular parameters investigated from the mixed cohort of healthy subjects and patients are shown in Table 2.1. The velocity component of FMD has not been consistently measured in previous studies so we didn't always measure this parameter, especially when time was a factor. The later studies in the Vascular and Inflammatory Diseases Research Unit, University of Dundee included velocity data, after it had been reported in the literature that this data provides important information in addition to the FMD percentage change (Padilla et al., 2009, Anderson et al., 2011).

Table 2.1 The demographics and key vascular parameters investigated in a mixed population of healthy subjects (HS) and patients (PT).

	HS & PT	***n***
Age (years)	37.7±20.2	(651)
Gender (M:F)	330:383	(713)
Systolic BP (mmHg)	123.5±17.7	(580)
Diastolic BP (mmHg)	74.8±11.9	(580)
Height (m)	1.63±0.14	(577)
Weight (Kg)	65.2±19.1	(584)
BMI (Kg/m^2)	24.5±5.6	(631)
ACh AUC (units)	187507±104610	(555)
SNP AUC (units)	110601±69530	(555)
FMD baseline (mm)	4.02±5.73	(486)
FMD peak (mm)	4.05±0.92	(487)
FMD change (%)	7.28±2.82	(489)
Integral baseline (m/s)	0.19±0.11	(366)
Integral post (m/s)	0.88±0.31	(366)
Integral change (%)	490±313	(366)
Maximum baseline (m/s)	0.88±0.30	(358)
Maximum post (m/s)	1.57±0.43	(358)
Maximum change (%)	87±51	(358)

Results are expressed as mean ± SD. HS: healthy subjects, PT: patients, BP: blood pressure, BMI: body mass index, ACh: acetylcholine, AUC: area under the curve, SNP: sodium nitroprusside, FMD: flow mediated dilatation.

2.2.1 Relationship between Skin Microvascular Function and FMD (All Study Data: Healthy Subjects and Patients)

The correlations between skin microvascular function and FMD are listed in Table 2.2. There were significant positive correlations between ACh AUC and FMD percentage change and between ACh AUC and the integral velocity percentage change (Table 2.2, Figure 2.4 and Figure 2.5). There were also significant positive correlations between SNP AUC and integral velocity change and between SNP AUC and the post maximum velocity (Table 2.2).

Table 2.2 Pearson correlations between skin microvascular function and FMD and the FMD velocity in a mixed population of healthy subjects (HS) and patients (PT).

	r	**r^2**	***p***	***n***
ACh AUC and FMD change	0.11	0.01	**0.048**	(336)
SNP AUC and FMD change	-0.01	0.00	0.92	(336)
ACh AUC and Integral post	0.10	0.01	0.09	(267)
SNP AUC and Integral post	0.12	0.02	**0.048**	(268)
ACh AUC and Integral change	0.25	0.07	**<0.001**	(266)
SNP AUC and Integral change	0.10	0.01	0.12	(267)
ACh AUC and Maximum post	0.10	0.01	0.10	(260)
SNP AUC and Maximum post	0.12	0.02	**0.049**	(261)
ACh AUC and Maximum change	0.05	0.00	0.43	(260)
SNP AUC and Maximum change	-0.00	0.00	0.96	(261)

ACh: Acetylcholine, AUC: area under the curve, FMD: flow mediated dilatation, SNP: sodium nitroprusside

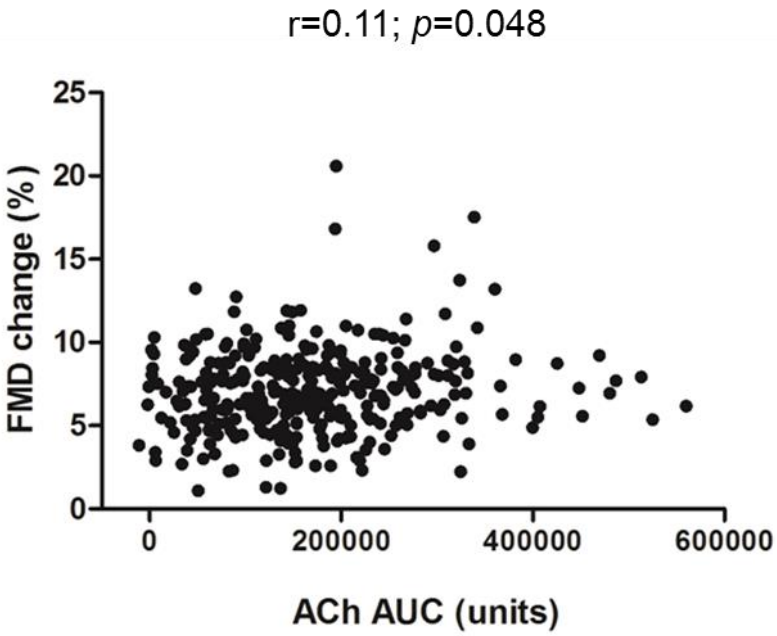


Figure 2.4 The relationship between ACh AUC and FMD percentage change in a mixed population made up of healthy subjects and patients (*n*=336).

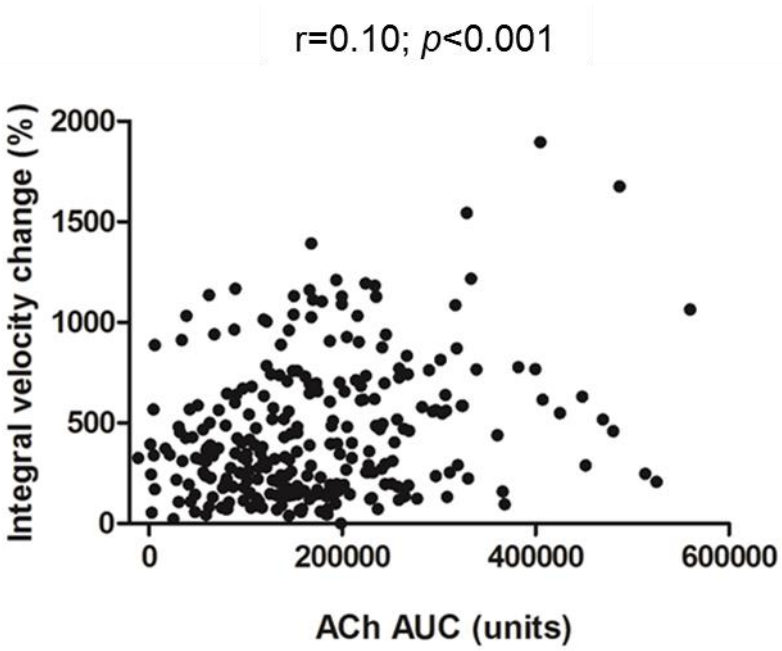


Figure 2.5 The relationship between ACh AUC and integral velocity percentage change in a mixed population made up of healthy subjects and patients (*n*=266).

2.2.2 Relationship between Skin Microvascular Function and FMD (Healthy Subjects only)

The healthy subjects included in the dataset were studied separately from the patients to further investigate the relationship between skin microvascular function and FMD in

subjects free from disease. The demographics and main vascular parameters for the healthy subjects are displayed in Table 2.3.

Table 2.3 The demographics and key vascular parameters investigated in a group of healthy subjects (HS).

	HS	***n***
Age (years)	32.5±19.3	(410)
Gender (M:F)	200:213	(413)
Systolic BP (mmHg)	118.9±15.2	(358)
Diastolic BP (mmHg)	72.9±11.4	(358)
Height (m)	1.60±0.16	(342)
Weight (Kg)	60.9±19.5	(351)
BMI (Kg/m^2)	23.8±5.5	(398)
ACh AUC (units)	205005±104712	(332)
SNP AUC (units)	118373±66696	(332)
FMD baseline (mm)	3.76±0.81	(252)
FMD peak (mm)	4.03±0.83	(252)
FMD change (%)	7.61±3.25	(254)
Integral baseline (m/s)	0.15±0.09	(226)
Integral post (m/s)	0.92±0.35	(225)
Integral change (%)	596±321	(225)
Maximum baseline (m/s)	0.81±0.28	(222)
Maximum post (m/s)	1.58±0.49	(222)
Maximum change (%)	103±52	(222)

Results are expressed as mean ± SD. HS: healthy subjects, BP: blood pressure, BMI: body mass index, ACh: acetylcholine, AUC: area under the curve, SNP: sodium nitroprusside, FMD: flow mediated dilatation.

The correlations between skin microvascular function and FMD for the healthy subjects are listed in Table 2.4. There was a significant correlation between ACh AUC and FMD percentage change and between ACh AUC and integral velocity percentage change (Table 2.4, Figure 2.6 and Figure 2.7). All other correlations failed to reach significance.

Table 2.4 Pearson correlations between skin microvascular function and FMD and the FMD velocity in a group of healthy subjects (HS).

	r	**r^2**	***p***	***n***
ACh AUC and FMD change	0.16	0.03	**0.032**	(176)
SNP AUC and FMD change	-0.01	0.00	0.91	(176)
ACh AUC and Integral post	0.03	0.00	0.72	(172)
SNP AUC and Integral post	0.09	0.01	0.24	(173)
ACh AUC and Integral change	0.20	0.04	**0.008**	(172)
SNP AUC and Integral change	0.06	0.00	0.42	(173)
ACh AUC and Maximum post	0.06	0.00	0.46	(170)
SNP AUC and Maximum post	0.10	0.01	0.19	(171)
ACh AUC and Maximum change	-0.04	0.00	0.62	(170)
SNP AUC and Maximum change	-0.03	0.00	0.75	(171)

ACh: Acetylcholine, AUC: area under the curve, SNP: sodium nitroprusside, FMD: flow mediated dilatation

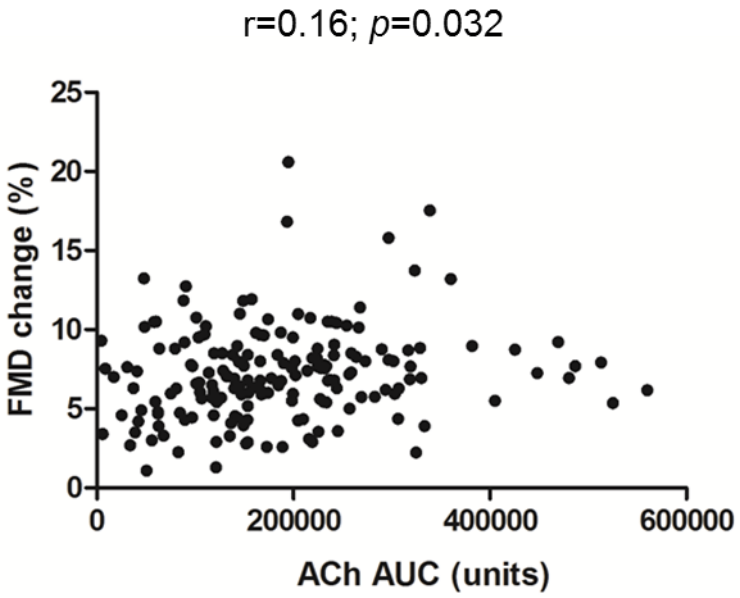


Figure 2.6 The relationship between ACh AUC and FMD percentage change in a population of healthy subjects (*n*=176).

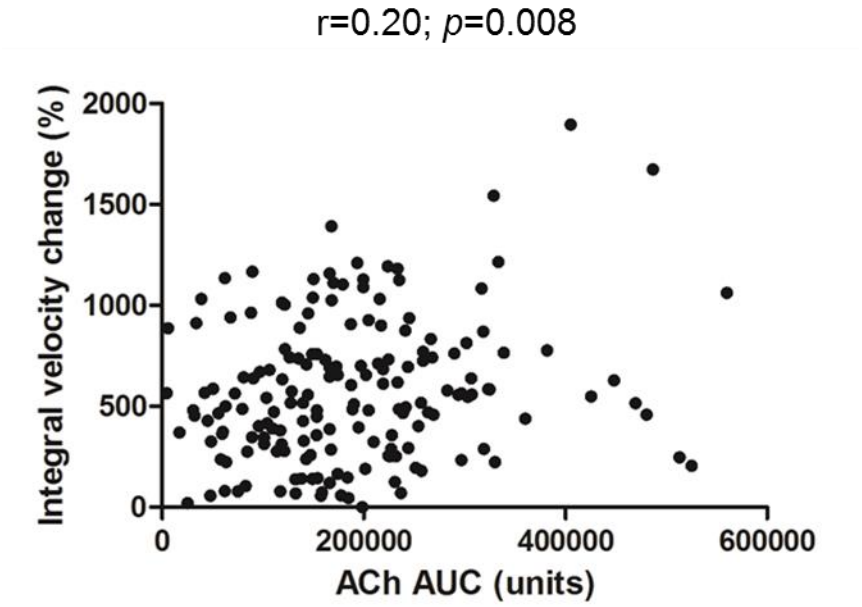


Figure 2.7 The relationship between ACh area under the curve (AUC) and integral velocity percentage change in a population of healthy subjects (*n*=172).

2.3 Discussion

Data obtained from several research studies conducted within the Vascular and Inflammatory Diseases Research Unit, University of Dundee provided an opportunity to establish whether skin microvascular function provides a marker of overall vascular function. This was specifically investigated by observing the relationship between skin microvascular function, measured by iontophoresis of ACh, and brachial artery velocity, the stimulus for FMD. A detailed regression analysis was not carried out as the main outcome was a direct comparison between the two measures.

Using the combined dataset made up of healthy subjects and patients, it was discovered there was a positive correlation between ACh AUC and FMD, as well as between ACh AUC and the change in the integral velocity. These results indicate a positive association between skin endothelial function and the macrocirculation.

It is well established that FMD is a good predictor of future CV events, but more recently the FMD velocity component itself has been identified as a significant

predictor of CV outcome. As described in chapter 1, brachial artery velocity has been shown to successfully predict CV outcome when the traditional FMD percentage change could not (Anderson et al., 2011). This new measure of microvascular function may provide a better tool for categorising individuals at risk of CVD, especially those individuals within a lower risk group (Padilla et al., 2009).

Recent studies suggest that shear stress should be calculated and corrected for in the FMD analysis response (Parker et al., 2009), based on the understanding that the FMD response relies on the brachial artery velocity shear stress stimulus. The velocity component of FMD is heavily influenced by many factors which include age, sex, brachial artery diameter and NO availability within the resistance vessels in the forearm (Pyke and Tschakovsky, 2005). The brachial artery diameter can have a considerable impact on the shear stress stimulus; if two vessels with different diameters have the same flow rate, the shear stress stimulus generated will be very different. This is because a smaller vessel will exhibit a much greater velocity and therefore a greater shear stress stimulus compared to a larger vessel with the same flow rate. In order to overcome these differences in shear stress stimulus, normalisation is recommended. However, it still remains unclear which method is the best for normalisation of the shear stress stimulus; both the peak shear response and the AUC shear response can be used, but it is not clear at present which method is most appropriate (Parker et al., 2009).

The results from the current study identify the velocity profile itself as an important parameter that can provide useful information independently of the FMD response. Of note, the correlation between ACh AUC and the integral velocity change was stronger than the relationship between ACh AUC and FMD. ACh AUC and integral velocity change both assess forearm microvascular function rather than macrovascular function,

which may explain why the relationship between these parameters was more closely associated.

As well as looking at a mixed cohort of healthy subjects and patients, the data collected from only the healthy subjects was analysed separately. It was decided not to analyse the patient data separately as the patients included in the dataset had varying levels of disease. In healthy subjects there was a significant positive correlation between ACh AUC and FMD percentage change and between ACh AUC and integral velocity change. These results indicate that, in a low risk population, skin microvascular function can provide useful information that can be related to the macrovasculature and overall vascular function. Like in the mixed patients and healthy subjects analysis, the ACh AUC had a stronger association with the integral velocity change compared with FMD change, further evidence that brachial artery velocity is an important component of the FMD test.

The findings from this part of the study suggest that skin microvascular function could provide a useful marker of overall vascular health since it was shown that skin microvascular function in the forearm positively correlates with the microvascular velocity component of FMD. Therefore, it appears that assessment of the skin microcirculation could provide an alternative, valuable method for determining general vascular function in addition to traditional FMD in healthy subjects, as well as a combined cohort of healthy subjects and patients. Furthermore, the assessment of brachial artery velocity should be included as part of the FMD test, after recent evidence has shown that this marker provides insight into CV risk stratification and predict CV outcome (Huang et al., 2007, Anderson et al., 2011).

The following chapters will provide details of an alternative test for assessment of skin microvascular function using FLPI in combination with PORH, which offers a quicker, simpler test compared to the current gold standard non-invasive assessment, FMD.

Chapter 3

The Development of a Post Occlusive Reactive Hyperaemia Protocol with FLPI

This chapter will describe the experiments performed to establish the most reproducible method to assess skin microvascular endothelial function using PORH with FLPI and will provide the results after each stage. Firstly, the protocols used to assess cuff position and measurement site will be addressed and, secondly the protocols used to investigate the effects of skin heating on PORH will be detailed.

3.1 Cuff Position and Measurement Site at the Forearm and Reproducibility Testing

3.1.1 Methods

This part of the study involved the development of a reproducible PORH protocol to assess endothelial function in combination with the FLPI (moorFLPI, Moor Instruments Ltd, Axminster, Devon, UK). The following measurement conditions were investigated to identify which conditions provided the most reproducible results:

- Cuff position (upper arm/lower arm)
- Measurement site at the forearm (proximal location/distal location)

In the skin, PORH is most commonly performed using an upper arm cuff with blood flow recorded at the forearm or finger (Roustit and Cracowski, 2013), but the test can also be performed using a lower arm cuff. The position of the cuff can affect the

hyperaemic response; a greater PORH response has been observed with an upper arm cuff position, which may be due to occluding a greater surface area and muscle mass, and stimulating a greater flow following the recruitment of more resistance vessels (Roustit et al., 2010, Pinto, 2007). Although a greater PORH response is typically elicited using an upper arm cuff it was important to compare it with a lower arm cuff position to see which method proved the most reproducible, as a higher PORH response may not necessarily result in better reproducibility.

Owing to the heterogeneity of skin blood flow, two measurement sites on the forearm (proximal and distal) were chosen to assess changes in skin blood flow in response to the reactive hyperaemia stimulus. Monitoring skin blood flow at two smaller measurement sites, rather than one larger measurement site on the forearm, enabled a specific location on the forearm to be determined as the most reproducible measurement site.

Laser Speckle Contrast Imaging Recordings

Forearm cutaneous perfusion measurements were recorded using the moorFLPI system (Figure 3.1), with a wavelength of 785 nm. The sampling frequency was 25Hz and the time constant was 1.0 second. The distance between the laser head and the skin surface was set to 30 cm. Skin blood flow was measured in up two regions of interest (ROI) depending on what measurement conditions were investigated (upper arm cuff: proximal and distal sites; lower arm cuff: distal site). The size of each ROI was 20 mm^2.

Figure 3.1 Set up of FLPI with an upper arm blood pressure cuff for PORH.

Study Procedures

Subjects were instructed to fast and refrain from caffeine, alcohol and tobacco for at least 2 hours before testing. Subjects were positioned comfortably in a supine position in a temperature controlled room (23-25ºC) and underwent an acclimatisation period of 10 minutes before testing began.

Study Participants

Subjects were recruited from the local student and staff population at the University of Dundee, Ninewells Hospital Campus through poster advertisements displayed around Ninewells Hospital. All subjects who participated in the study were considered healthy and free of recognised symptomatic CV and metabolic diseases. Subjects were provided with a study information sheet at least 24 hours prior to testing and were given the

opportunity to have any questions answered before the study visit. Written informed consent was obtained from each subject prior to participation in the study. The study was approved by the University of Dundee Research Ethics Committee.

Cuff Position and Measurement Site at the Forearm

As this project was assessing FLPI as a new technique for PORH, no formal power calculation was available to determine the sample size, and from previous pilot work carried out in the Vascular and Inflammatory Diseases Research Unit, University of Dundee using laser Doppler technology, a sample size of n=15 was chosen. Furthermore, the reproducibility of PORH using LSCI has previously been investigated using a subject size of n=14 (Roustit et al., 2010). Fifteen healthy volunteers (9 female and 6 male (mean (±SD)) age 30.3 (± 9.4) years, height 166.8 (± 7.3) centimetres and weight 66.3 (± 14.6) kilograms) were studied on 2 occasions one day apart to investigate the following combinations of cuff position and measurement site during a PORH protocol (Figure 3.2, Figure 3.3 and Figure 3.4):

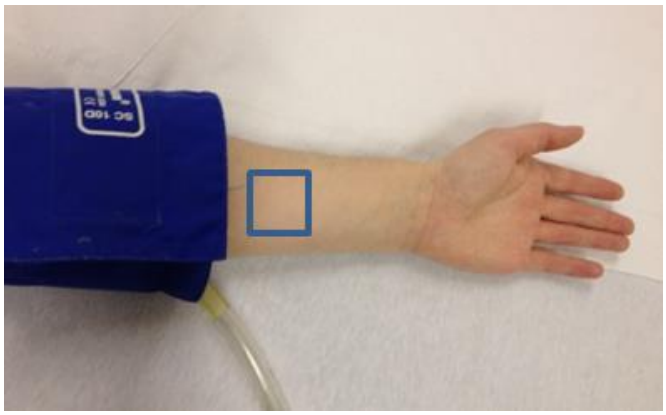


Figure 3.2 Lower arm cuff/distal measurement site (LD) (studied on day 1).

When investigating the lower arm cuff position, only the distal measurement site could be monitored as the proximal measurement site was situated at the same location as the lower arm cuff.

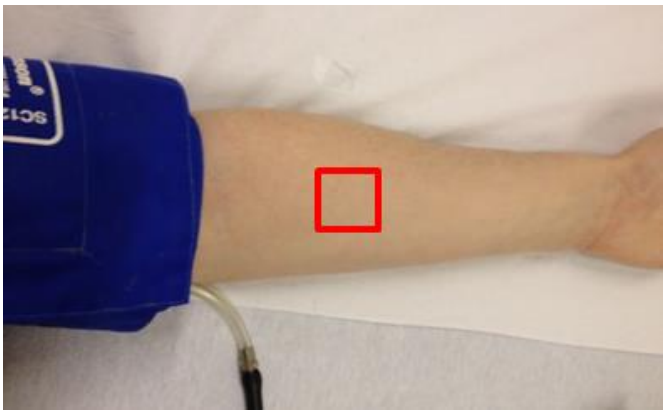


Figure 3.3 Upper arm cuff/proximal measurement site (UP) (studied on day 2).

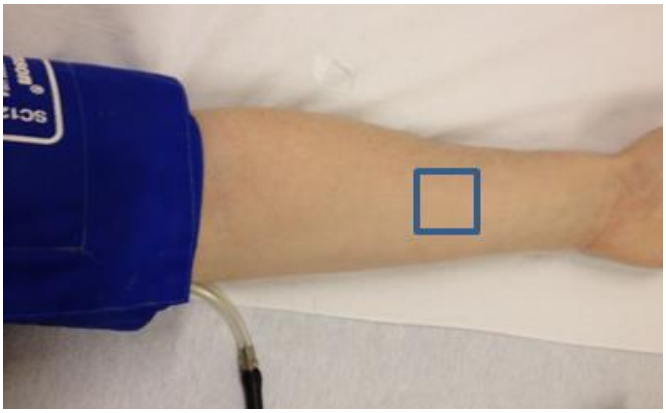


Figure 3.4 Upper arm cuff/distal measurement site (UD) (studied on day 2).

Post Occlusive Reactive Hyperaemia

A blood pressure cuff (SC10D/SC12, Hokanson Bellevue, Washington, USA) was placed around the right lower or upper arm, depending on which cuff position and measurement site were being investigated. The distal measurement site was positioned 10cm from the base of the hand and the proximal measurement site was positioned 5cm from the crease of the elbow on the volar surface of the forearm. Baseline forearm skin perfusion was monitored for 1 minute prior to a 5 minute cuff occlusion, obtained by inflating a blood pressure cuff suprasystolically to a pressure of 200 mmHg. Skin perfusion was monitored during this period of ischaemia and continued to be assessed for a further 2 minutes following cuff release. Forearm skin temperature was recorded at the distal measurement site using a non-contact infra-red thermometer (Mini IR

Thermometer, RS Components Ltd, Corby, Northants, UK) before and after the PORH protocol. The PORH response was calculated using equation 3.1:

$$PORH(\%) = \left(\frac{Peak\ skin\ perfusion - baseline\ skin\ perfusion}{baseline\ skin\ perfusion}\right) \times 100 \quad \textbf{3.1}$$

Reproducibility Testing of the PORH Protocol

Ten of the 15 healthy volunteers studied were invited back for reproducibility testing of the PORH protocol with FLPI. Ten subjects had the upper arm cuff position with the distal and proximal measurement sites re-tested and 5 subjects had the lower arm cuff position and the distal measurement site re-tested, using the same experimental conditions as described above. PORH is most commonly performed using an upper arm cuff occlusion so was the preferred method to use for reproducibility testing. However, 5 subjects were invited back for reproducibility testing with the lower arm cuff occlusion to see how this method compared with upper arm cuff occlusion.

Data Analysis

Data is expressed as the mean ± standard deviation (SD). The reproducibility of the PORH test with FLPI was determined using percentage variance; the absolute mean difference in PORH percentage change between the initial visit and reproducibility visit was divided by the combined mean PORH percentage change from the initial and reproducibility visits. Students paired *t* test and Pearson correlation were selected for statistical analyses. For all statistical tests a p value of <0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS 18 (SPSS Inc., Illinois, Chicago, USA).

3.1.2 Results

Cuff Position and Measurement Site at the Forearm and Reproducibility Testing

Three different measurement protocols were investigated on 2 occasions one day apart to establish which method provided the most reproducible assessment of endothelial function by performing PORH measured by FLPI (Figure 3.5); 1. lower arm cuff and distal measurement site (LD), 2. upper arm cuff and proximal measurement site (UP), and 3. upper arm cuff and distal measurement site (UD). The results for each measurement protocol for all study subjects at visit 1 are shown in Table 3.1.

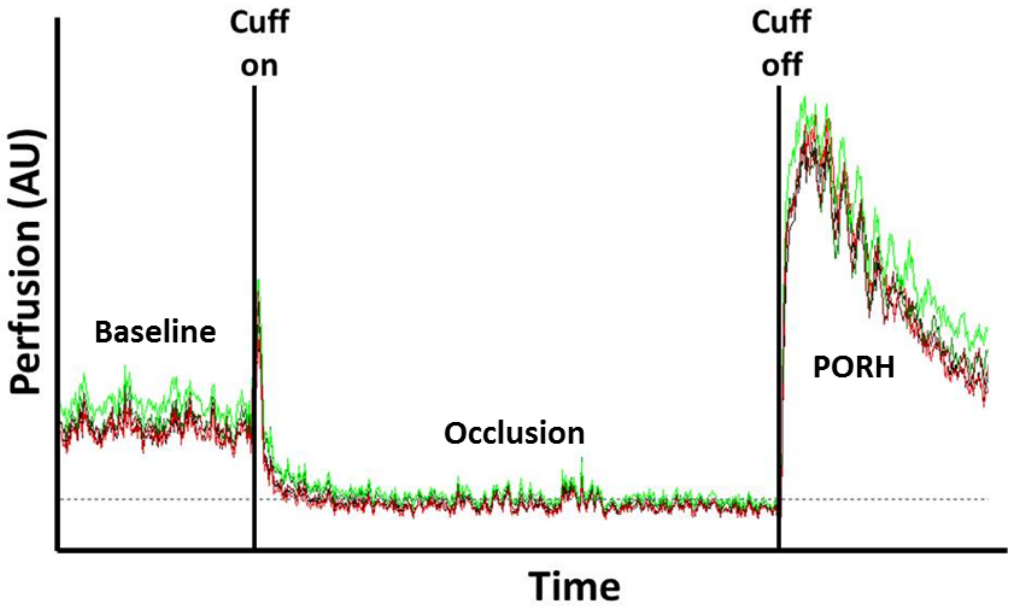


Figure 3.5 An example of skin perfusion recorded at baseline, during arterial occlusion and immediately after cuff release to assess the transient increase in blood flow – post occlusive reactive hyperaemia (PORH).

Table 3.1 Blood flow parameters measured with FLPI for each measurement set up at visit 1 (*n*=15).

	LD		UP		UD	
Baseline (PU)	64.6±22.7	(35.2)	59.3±18.6	(31.3)	64.3±19.3	(29.9)
Occlusion (PU)	27.4±11.4	(41.4)	19.5±7.1	(36.5)	24.2±9.9	(41.0)
Peak (PU)	298.6±121.9	(40.8)	239.8±68.9	(28.7)	263.5±88.4	(33.5)
PORH (%)	369.9±110.8	(29.9)	310.3±74.4	(23.9)	312.4±88.3	(28.2)

Results are expressed as mean ± SD with the coefficient of variation in brackets. LD: lower arm cuff and distal measurement site, UP: upper arm cuff and proximal measurement site, UD: upper arm cuff and distal measurement site, PU: perfusion units, PORH: post occlusive reactive hyperaemia.

There were no significant differences in PORH response between visit 1 and visit 2 for either UD, UP or LD (

Figure 3.6, Figure 3.7, Figure 3.9 and Table 3.2). Bland-Altman plots were produced for the UP and UD measurement set ups to show the mean-difference for the PORH responses from visit 1 and visit 2 (Figure 3.8 and Figure 3.10). The reproducibility of the test was measured by comparing results from visit 1 and visit 2. The most reproducible PORH results were obtained with the UD measurement set up. The UP measurement set up was the second most reproducible and the LD measurement set up was the least reproducible (Variance 8.8%; 17.4%; 31.1% respectively) (Table 3.2).

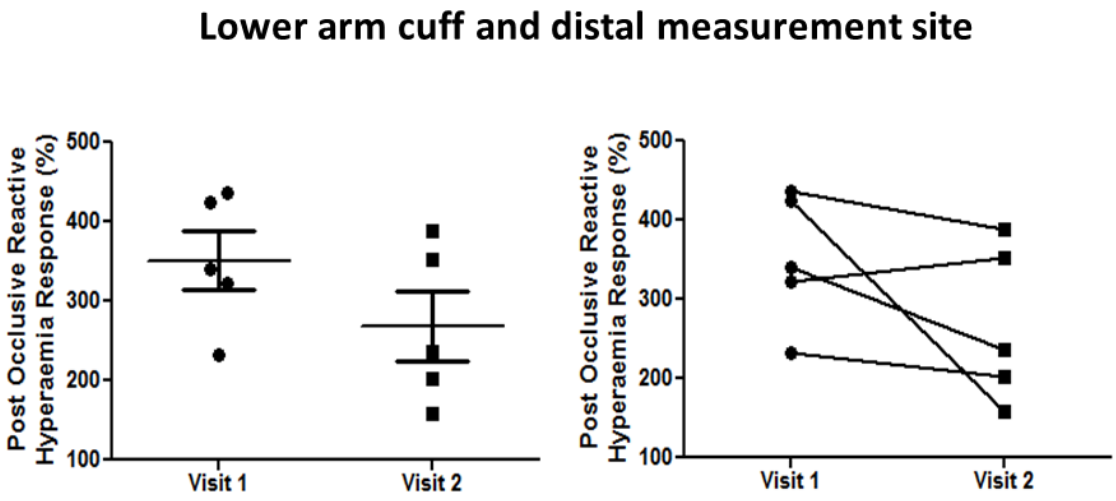


Figure 3.6 PORH response measured with FLPI at visit 1 and visit 2 using the lower arm cuff and distal measurement site presented in two ways (Variance 31.1%) (n=5).

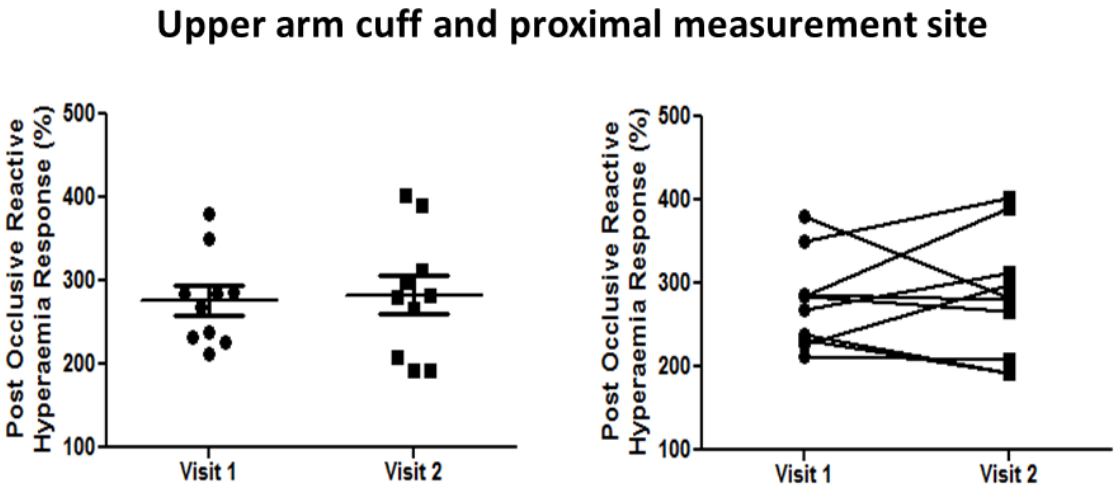


Figure 3.7 PORH response measured with FLPI at visit 1 and visit 2 using the upper arm cuff and proximal measurement site presented in two ways (Variance 17.4%) (*n*=10).

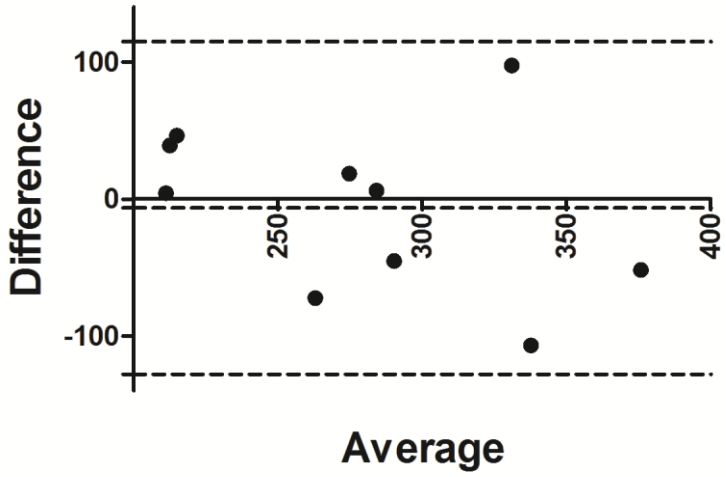


Figure 3.8 Bland-Altman plot for the upper arm cuff and proximal measurement site; showing the mean-difference for the PORH responses from visit 1 and visit 2. The upper and lower dotted lines on the graph represent the upper and lower 95% limits of agreement.

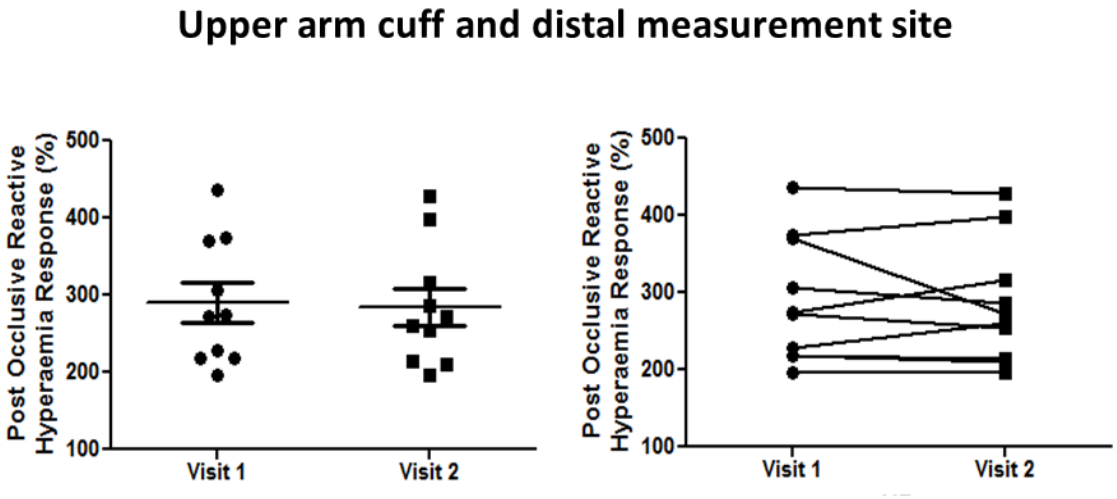


Figure 3.9 PORH response measured with FLPI at visit 1 and visit 2 using the upper arm cuff and distal measurement site presented in two ways (Variance 8.8%) (*n*=10).

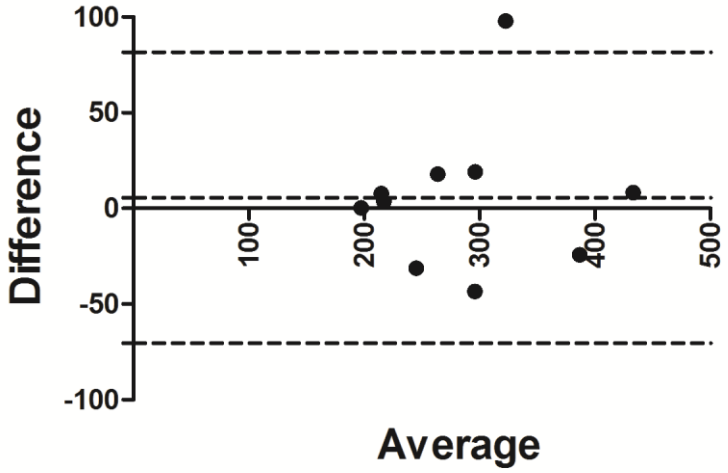


Figure 3.10 Bland-Altman plot for the upper arm cuff and distal measurement site; showing the mean-difference for the PORH responses from visit 1 and visit 2. The upper and lower dotted lines on the graph represent the upper and lower 95% limits of agreement.

Table 3.2 PORH response and the percentage variability measured with FLPI for each measurement set up at visit 1 and visit 2 (*n*=10 for upper arm cuff and distal site and upper arm cuff and proximal site and n=5 for lower arm cuff and distal site).

Cuff Position	Measurement Site	PORH% Change Visit 1	PORH % Change Visit 2	% Variation	*p* Value
Lower Arm	Distal	351.3±82.9	267.6±98.8	31.1	0.09
Upper Arm	Proximal	276.3±54.1	282.7±73.9	17.4	0.37
Upper Arm	Distal	289.8±80.8	284.2±77.8	8.8	0.33

Results are expressed as mean ± SD.

3.2 Effects of Forearm Skin Temperature on Skin Perfusion and Post Occlusive Reactive Hyperaemia

3.2.1 Methods

Skin temperature is known to affect skin blood flow and therefore this factor was taken into consideration for the PORH protocol. To minimise the effect of skin temperature on vascular function, additional experiments were conducted using a skin heater (SH02 Skin Heater, Moor Instruments Ltd, Axminster, Devon, UK) to enable forearm skin temperature to be standardised between subjects prior to PORH (Figure 3.11). The distal measurement site was found to be the most reproducible measurement site in the previous set of PORH experiments without heating and was therefore selected as the site for skin heating. A small, water filled, circular chamber was attached to the surface of the skin using an adhesive ring (IAD, Moor Instruments Ltd, Axminster, Devon, UK). The water in the chamber was heated to a temperature of 35°C in all subjects and skin perfusion was assessed using FLPI. A skin temperature of 35°C was chosen as heating to this temperature has previously been shown to have no significant effect on skin blood flow (Beed et al., 2009).

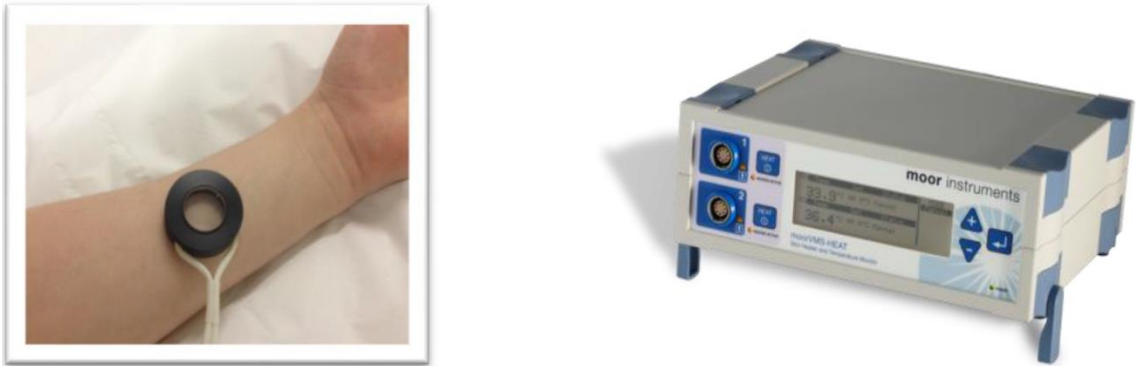


Figure 3.11 Skin heating chamber at the distal measurement site (left) and heating module (right).

Baseline Perfusion with Skin Heating

Eight healthy volunteers (5 female and 3 male (mean (±SD) age 29.6 (± 9.2), height 168.8 (± 6.5) centimetres and weight 65.0 (± 6.1) kilograms)) had baseline forearm skin

perfusion assessed using FLPI at both the proximal and distal locations. The heating chamber was placed at the distal site (the most reproducible site), and blood flow was monitored with unheated water and when water had reached a temperature of 35°C. Skin perfusion at the distal site was first recorded with unheated water for 5 minutes, then the skin heater was switched on and once a temperature of 35°C was reached skin perfusion was monitored for an additional 5 minutes. By monitoring skin perfusion with unheated water and heated water at the distal site, changes in skin perfusion as a result of skin heating could be assessed. Simultaneously, skin perfusion was also measured at the proximal site on the forearm (without any heating) to see if there were any systemic effects as a result of heating the skin at the distal forearm site.

Post Occlusive Reactive Hyperaemia with Skin Heating

The next step was to combine skin heating at the distal site with the PORH protocol to assess how this would affect the reproducibility of the test. Ten healthy volunteers (7 female and 3 male (mean (±SD)) age 32.6 (± 11.3), height 169.7 (± 6.6) centimetres and weight 68 (± 9.1) kilograms) had their skin perfusion assessed, using FLPI to monitor the effect of heating the forearm skin at the distal measurement site before and during PORH, on 2 consecutive days (Figure 3.12). Subjects followed the same pre-test guidelines and underwent the same acclimatisation period described in section 2.1.2. The heating protocol detailed earlier in this section used to standardise subjects forearm skin temperature was adapted slightly; skin perfusion with unheated water was recorded for 4 minutes rather than 5 minutes. This part of the protocol was reduced as it was found that there was no difference in perfusion from 4 minutes to 5 minutes. Baseline forearm skin perfusion was monitored for 2 minutes prior to a 5 minute cuff occlusion period, obtained by inflating a blood pressure cuff suprasystolically to a minimum pressure of 200 mmHg. Skin perfusion was monitored during this period of ischaemia and continued to be assessed for a further 4 minutes following cuff release. Skin

perfusion was measured for an extended period of time post cuff to enable the PORH response to be monitored over a longer time period. Skin perfusion was also measured simultaneously throughout the test at the proximal site of the forearm (without heating).

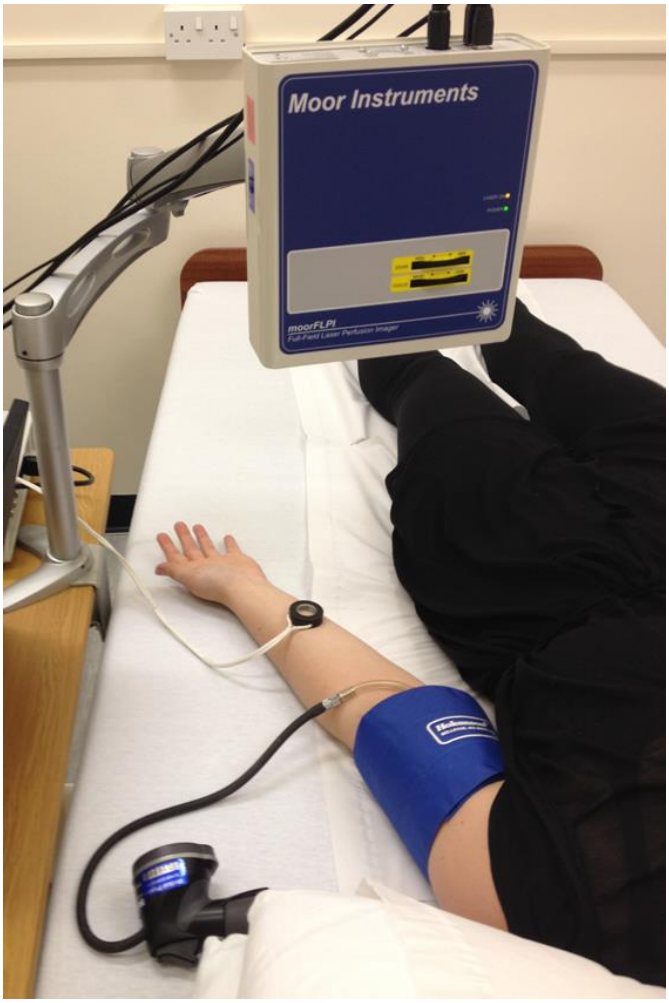


Figure 3.12 Set up of FLPI with an upper arm blood pressure cuff and the heating chamber.

3.2.2 Results

Effects of Forearm Skin Temperature on Skin Perfusion and Post Occlusive Reactive Hyperaemia

Forearm skin temperature was recorded at visit 1 and visit 2 and ranged from 26.9°C to 35.6°C across the study population (Table 3.3).

Table 3.3 Baseline skin temperature at visit 1 (*n*=10) and visit 2 (*n*=10).

Baseline Skin Temperature (°C)	Visit 1	Visit 2
Minimum	26.9	29.8
Maximum	35.6	33.8
Mean	31.4±1.9	31.8±1.3

Mean results expressed ± SD.

The relationship between baseline skin temperature and blood flow was investigated at visit 1. However, it was found that there were no significant correlations between baseline skin temperature and baseline perfusion, peak perfusion, or PORH (Table 3.4).

Table 3.4 Pearson correlations between baseline skin temperature and blood flow parameters for each of the measurement protocols at visit 1 (*n*=15).

	LD			UP			UD		
	r	r^2	*p*	r	r^2	*P*	r	r^2	*p*
Baseline Skin Temperature and Baseline Flow	0.38	0.14	0.25	-0.74	0.55	0.83	0.09	0.01	0.78
Baseline Skin Temperature and Peak Flow	0.24	0.06	0.48	0.48	0.23	0.89	0.20	0.04	0.55
Baseline Skin Temperature and PORH	-0.46	0.21	0.89	0.28	0.08	0.41	0.29	0.09	0.38

Additional experiments showed that compared with unheated water, water heated to 35°C had no effect on skin perfusion at baseline as measured by FLPI (83.4±22.5 PU VS. 86.1±25.0 PU, *p*=0.30). Heating of the forearm skin to 35°C prior to PORH was then carried out to assess the reproducibility of the test using the UD measurement set up, as this was found to be the most reproducible measurement protocol. There was no significant difference in PORH response between measurements at two different visits one day apart (155.1±44.6 % VS. 183.8±103.2 % Variance 41.2%, *p*=0.16) (Figure 3.13). Although there was no significant change in PORH response following skin heating to 35°C, there was a change in the reproducibility of the test. Heating the skin before and during PORH resulted in poorer reproducibility when compared with the

PORH test without any skin heating. The percentage variability when the PORH test was performed with heating was almost 5 times higher than that calculated when the PORH test was performed without forearm heating to 35°C (Variance: PORH with skin heating 41.2% VS. PORH without heating 8.8%). Furthermore, there was a considerable increase in the coefficient of variation between the 2 visits; from 30% at visit 1 to 59% at visit 2.

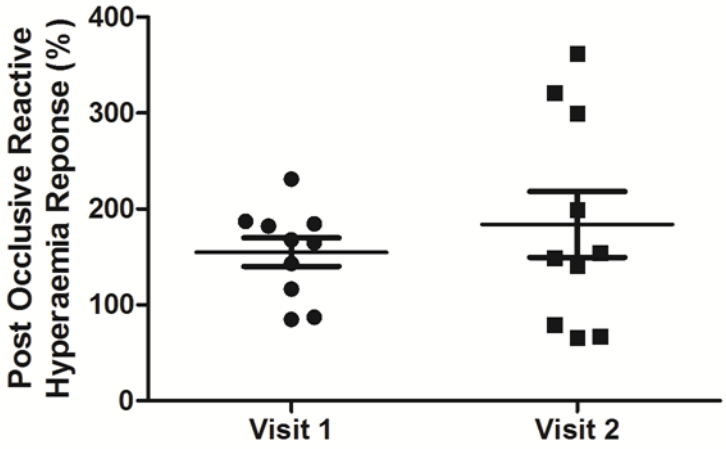


Figure 3.13 PORH response using an upper arm cuff in combination with forearm skin heating at the distal measurement site to a temperature of 35°C on two separate occasions (Variance 41.2%) (*n*=10).

The results from the developmental phase of the study indicate that the most reproducible method of PORH to use with FLPI is the UD measurement set up without heating of the forearm skin. These conditions were used for the next stage of the study when PORH with FLPI was compared with EndoPAT in two different age groups.

Chapter 4

Comparison of Techniques used to Assess Microvascular Endothelial Function

4.1 Methods

Thirty healthy volunteers were recruited into two groups from the University of Dundee staff and student population. Group 1 included subjects aged between 18 and 30 years of age and group 2 included subjects aged between 40 and 70 years of age. All subjects who participated in the study were considered healthy and free of recognised symptomatic CV and metabolic diseases. These two age groups were selected to assess the effect of age on microvascular endothelial function as it is well known that with aging many structural and functional changes occur in the microcirculation and also endothelium-dependent function has previously been shown to be blunted with increasing age (Taddei et al., 1995, Gerhard et al., 1996).

Subjects followed the same pre-test guidelines and underwent the same acclimatisation period as mentioned in section 2.1.2. BP was measured automatically three times in the right arm, 2cm above the antecubital fossa (Omron M6, OMRON Healthcare Europe, Hoofddorp, The Netherlands).

4.1.1 Post Occlusive Reactive Hyperaemia with FLPI

The finalised PORH protocol was performed using an aneroid sphygmomanometer (SC12, Hokanson Bellevue, Washington, USA) placed around the upper left arm above the elbow, with regions of interest placed on the volar forearm at the proximal and distal

measurement sites. Skin perfusion at these sites was assessed with FLPI using the same PORH protocol that was performed in section 3.2.1, but without skin heating at the distal measurement site as heating failed to improve PORH reproducibility. Although the distal measurement site was shown to be more reproducible, it was still possible to measure skin perfusion at the proximal measurement site, so it was decided to collect the additional data as well. Forearm skin temperature was measured at the proximal and distal measurement sites before and after the PORH protocol (Mini IR Thermometer, RS Components Ltd, Corby, Northants, UK). Besides PORH, another blood flow parameter, total hyperaemic response (THR), was also calculated for each of the groups using the following equation:

$$THR(units) = AUC\ of\ the\ hyperaemic\ response - baseline\ blood\ flow \quad \textbf{4.1}$$

4.1.2 Peripheral Arterial Tonometry

Peripheral Arterial Tonometry (PAT) was chosen as an alternative method to assess endothelial function in the study population because, similarly to PORH measured using FLPI, this technique also measures endothelial function in the resistance blood vessels of the microcirculation. PAT is also the only FDA approved measure of endothelial function and is increasingly being used in clinical research.

Twenty minutes after the PORH test using FLPI, the PAT test was performed using the EndoPAT device (Itamar Medical Ltd, Caesarea, Israel) following the manufacturer's guidelines. An aneroid sphygmomanometer (SC12, Hokanson Bellevue, Washington, USA) was placed around the upper left arm above the elbow and the fingertip plethysmography probes were placed on the index fingers of each hand. The pulse wave amplitude was recorded continuously by the device for the full 16 minute protocol. The

test began with a 5 minute baseline period, followed by 5 minutes with the cuff inflated to a suprasystolic BP (minimum 200 mmHg) and then a final 6 minute period of reactive hyperaemia after cuff release. The main outcome measure, RHI is calculated automatically by the device's proprietary software as the ratio of the pulse amplitude 90-150 seconds post cuff to the average baseline pulse amplitude. The result is divided by the ratio of the contralateral control finger and multiplied by a baseline correction factor, to account for the influence of basal vascular tone. In addition to RHI, the software automatically calculates two measures of arterial stiffness, Alx and Alx standardised to a heart rate of 75 beats per minute (Alx@75), which were also included in the data analysis.

4.1.3 Statistical Analysis

Students unpaired *t* test and Pearson correlation were selected for statistical analyses. For all statistical tests a *p* value of <0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS 18 (SPSS Inc., Illinois, Chicago, USA).

4.2 Results

4.2.1 Differences between the Groups: G1 vs. G2

Thirty healthy subjects were recruited into two age groups to compare the PORH protocol using FLPI with EndoPAT; Group 1 (G1) included subjects aged between 18 and 30 years and Group 2 (G2) included subjects aged between 40 and 70 years. The baseline characteristics of all subjects are shown in Table 4.1.

Table 4.1 Baseline characteristics of healthy subjects; Group 1 (18-30 years) (*n*=15) and Group 2 (40-70 years) (*n*=15).

Characteristics	Group 1	Group 2	*p* value
Age (years)	25.5±2.1	50.8±5.9	<0.001
Sex male: female	8:7	7:8	
Height (m)	1.71±0.1	1.68±0.1	0.38
Weight (Kg)	69.5±10.9	72.9±12.5	0.43
BMI (Kg/m^2)	23.7±2.8	25.7±3.8	0.10
Systolic BP (mmHg)	112.6±8.2	121.9±15.3	0.023
Diastolic BP (mmHg)	63.8±6.5	73.0±7.1	<0.001
Heart rate (bpm)	65.6±7.4	60.5±8.6	0.045
Mean arterial pressure (mmHg)	80.1±6.4	89.3±9.4	0.001
UP PORH (%)	237.8±74.1	240.5±85.8	0.46
UD PORH (%)	228.9±74.2	230.2±86.6	0.48
UP THR (units)	16191±4031	16777±4015	0.35
UD THR (units)	16073±4395	17201± 703	0.23
UP Baseline Flow (PU)	126.1±25.9	148.6±37.1	0.032
UD Baseline Flow (PU)	124.6±25.4	150.4±41.6	0.025
UP Peak Flow (PU)	417.7±94.7	487.5±99.6	0.031
UD Peak Flow (PU)	402.9±90.8	477.1±107.0	0.025
RHI (units)	2.68±0.6	2.28±0.6	0.043
AIx (%)	-9.1±8.2	10.1±15.1	<0.001
AIx@75 (%)	-14.0±7.9	-0.1±13.8	0.001
Uncorrected PAT ratio (units)	3.91±1.6	3.40±1.3	0.18

Results are expressed as mean ± SD. BMI: Body Mass Index, BP: Blood Pressure, UP: upper arm cuff and proximal measurement site, PORH: Post Occlusive Reactive Hyperaemia, UD: upper arm cuff and distal measurement site, THR: Total Hyperaemic Response, PU: Perfusion Units, RHI: Reactive Hyperaemia Index, AI: Augmentation Index, AI@75: Augmentation Index normalised to a heart rate of 75 beats per minute.

There were significant differences between G1 and G2 for systolic BP, diastolic BP, heart rate and mean arterial pressure. All of these characteristics were significantly

lower in the younger G1 except for heart rate which was lower in the older G2 (Table 4.1).

There were no significant differences between G1 and G2 for PORH or THR using either the UD or UP measurement set ups (Table 4.1). However for both the UD and UP measurement set ups, there were significant differences between G1 and G2 for baseline and peak blood flow; for both parameters G2 had higher blood flow than G1 (Table 4.1).

There were significant differences for all measured parameters from the EndoPAT device; RHI was significantly higher in G1 compared to G2 and AIx and AIx@75 were significantly lower in G1 compared to G2 (Table 4.1). The uncorrected PAT ratio calculated only for the arm with the occlusion was not significantly different between G1 and G2 (Table 4.1).

4.2.2 All Study Data: G1 and G2 (*n*=30)

G1 and G2 study data was grouped together to look at correlations within a larger sample size of healthy subjects and over a broader age range. There were significant positive correlations between age and SBP (Figure 4.1), DBP (Figure 4.2), proximal baseline flow (Figure 4.3), distal baseline flow (Figure 4.4), AIx (Figure 4.5), AIx@75 and a significant negative correlation between age and RHI. There were no significant correlations found between age and PORH response at either the proximal or distal measurement site, however there was a trend towards a negative correlation between SBP and PORH response for both measurement sites (Table 4.2). The relationship between SBP, DBP and BMI with PORH and RHI was also investigated. There was a significant negative correlation between DBP and RHI, but all other correlations failed to reach significance (Table 4.2).

Table 4.2 Pearson correlations between age, blood pressure and different blood flow parameters across all study volunteers from Group 1 and Group 2 (n=30).

	r	r^2	*p* value
Age and SBP (mmHg)	0.40	0.16	0.031
Age and DBP (mmHg)	0.53	0.28	0.003
Age and Proximal Baseline Flow (PU)	0.43	0.18	0.018
Age and Distal Baseline Flow (PU)	0.45	0.20	0.013
Age and UP PORH %	-0.10	0.01	0.61
Age and UD PORH %	-0.08	0.01	0.68
Age and AIx (%)	0.63	0.40	0.001
Age and AIx@75 (%)	0.55	0.30	0.002
Age and RHI (units)	-0.36	0.13	0.049
SBP and UP PORH %	-0.32	0.10	0.09
DBP and UP PORH %	-0.05	0.00	0.81
SBP and UD PORH %	-0.26	0.07	0.16
DBP and UD PORH %	-0.12	0.01	0.54
SBP and RHI	-0.29	0.09	0.12
DBP and RHI	-0.37	0.14	0.043
BMI and UP PORH %	0.05	0.00	0.79
BMI and UD PORH %	0.04	0.00	0.85
BMI and RHI	-0.24	0.06	0.19

BP: Blood Pressure, PU: Perfusion Units, UP: upper arm cuff and proximal measurement site, UD: upper arm cuff and distal measurement site, AIx: Augmentation Index, AIx@75: Augmentation Index normalised to a heart rate of 75 beats per minute, RHI: Reactive Hyperaemia Index, BMI: Body Mass Index.

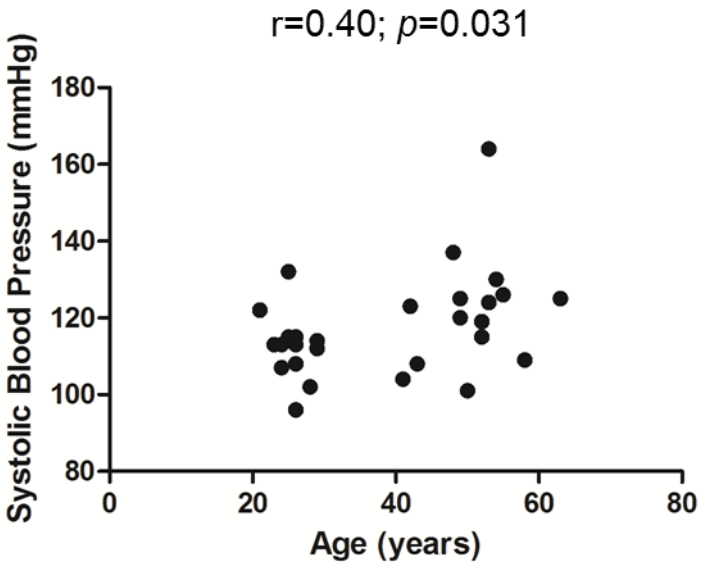


Figure 4.1 The relationship between age and systolic blood pressure across all study volunteers from Group 1 and Group 2 (*n*=30).

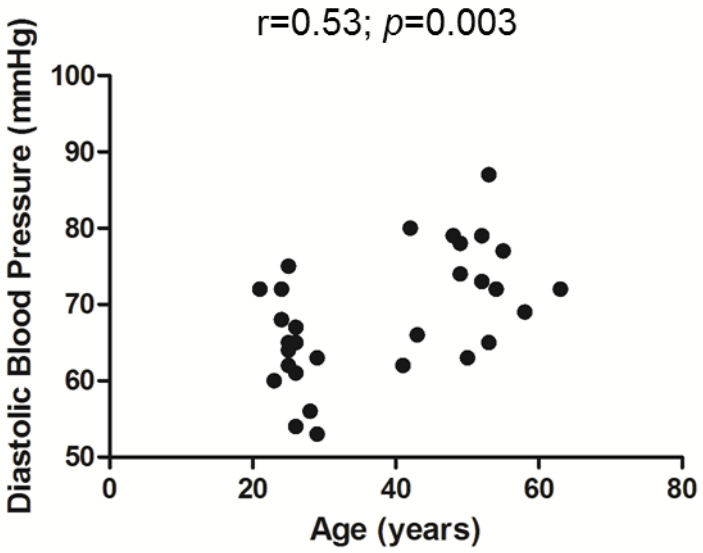


Figure 4.2 The relationship between age and diastolic blood pressure across all study volunteers from Group 1 and Group 2 (*n*=30**).**

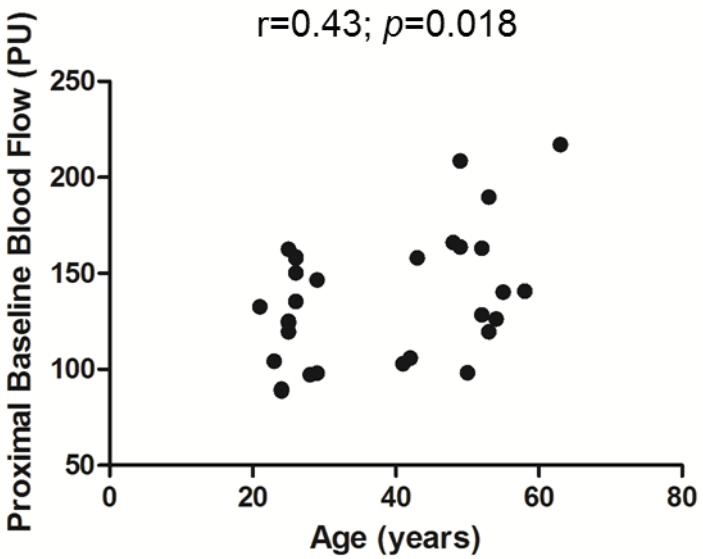


Figure 4.3 The relationship between age and proximal baseline blood flow across all study volunteers from Group 1 and Group 2 (*n*=30).

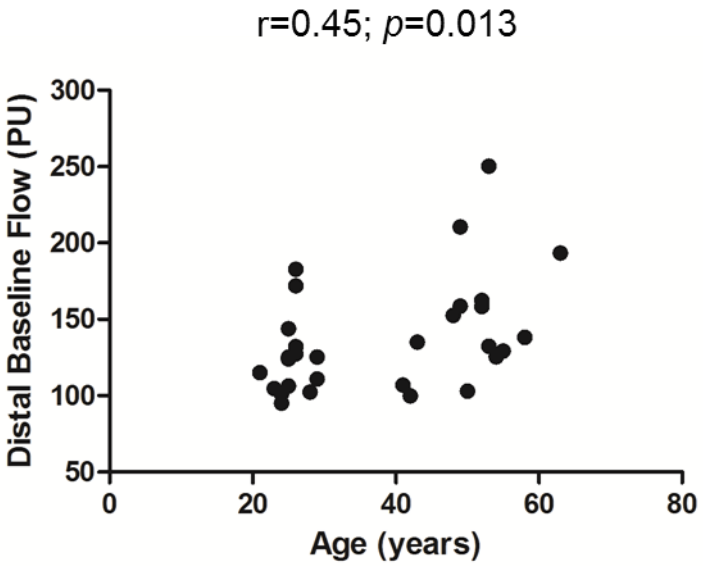


Figure 4.4 The relationship between age and distal baseline blood flow across all study volunteers from Group 1 and Group 2 (*n*=30).

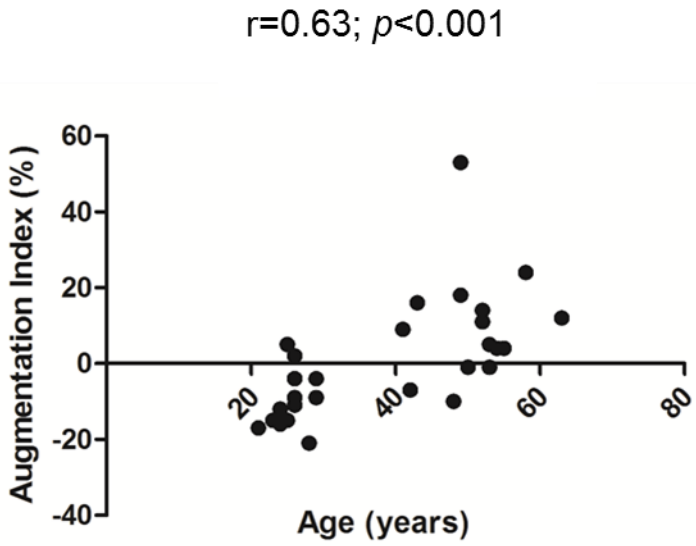


Figure 4.5 The relationship between age and augmentation index across all study volunteers from Group 1 and Group 2 (*n*=30).

There were no significant correlations noted between PORH responses at either measurement site with RHI (Table 4.3).

Table 4.3 Pearson correlations between the two methods of vascular function - PORH using FLPI and EndoPAT - across all study volunteers from Group 1 and Group 2 (*n*=30).

	r	**r^2**	***p* value**
UP PORH % and RHI	0.24	0.06	0.20
UD PORH % and RHI	0.17	0.03	0.36

UP: upper arm cuff and proximal measurement site, UD: upper arm cuff and distal measurement site, PORH: post occlusive reactive hyperaemia, RHI: reactive hyperaemia index.

There were significant positive correlations between the UD and UP measurement sites for each of the blood flow parameters assessed using FLPI; baseline flow, peak flow, PORH and THR (Table 4.4 and Figure 4.6).

Table 4.4 Pearson correlations between proximal and distal measurement sites for each of the blood flow parameters assessed using FLPI across all study volunteers from Group 1 and Group 2 (*n*=30).

Blood Flow Parameters	r	r^2	*p* value
Proximal and Distal Baseline Flow (PU)	0.87	0.75	<0.001
Proximal and Distal Peak Flow (PU)	0.86	0.73	<0.001
Proximal and Distal PORH (%)	0.90	0.81	<0.001
Proximal and Distal THR (PU)	0.78	0.62	<0.001

PU: Perfusion Units, PORH: Post Occlusive Reactive Hyperaemia, THR: Total Hyperaemic Response.

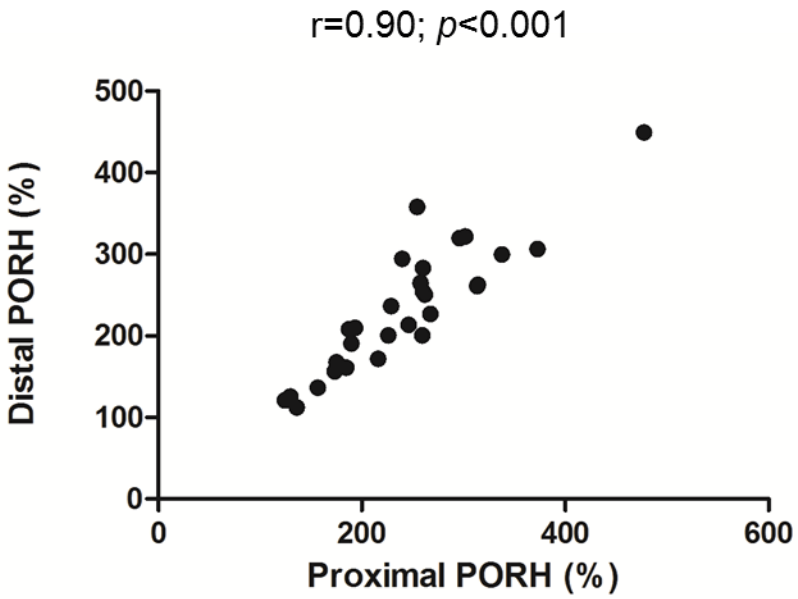


Figure 4.6 The relationship between PORH response at the proximal and the distal measurement sites using an upper arm blood pressure cuff across all study volunteers from Group 1 and Group 2 (*n*=30).

4.2.3 Within Age Group Correlations

There were no significant correlations found between age and the UP or the UD PORH responses within G1 (UP PORH r=-0.26, *p*=0.35, r^2=0.07; UD PORH r=0.04, *p*=0.88, r^2=0.17) or G2 (UP PORH r=-0.41, *p*=0.13, r^2=0.17; UD PORH r=-0.40, *p*=0.15, r^2=0.16). In G2, age and PORH were close to reaching significance, and Figure 4.7 and Figure 4.8 show that one subject is outwith the rest of the data set.

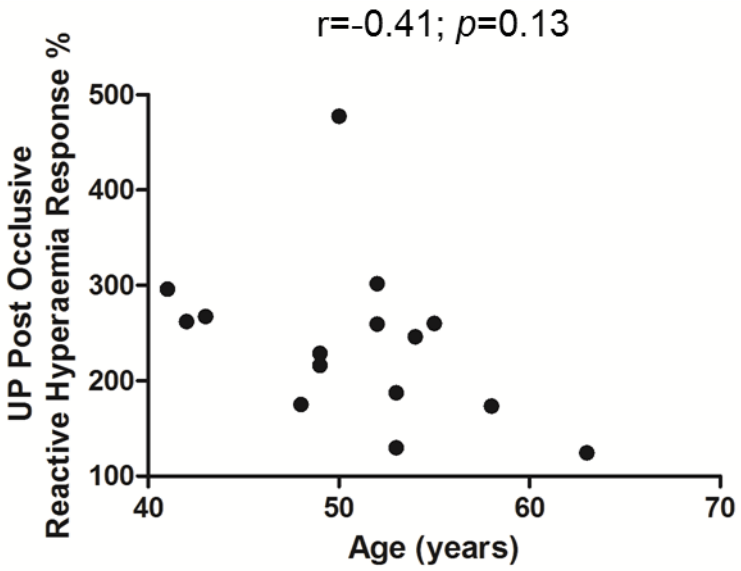


Figure 4.7 The relationship between age and PORH response in Group 2 measured with FLPI using an upper arm cuff and the proximal measurement site (*n*=15).

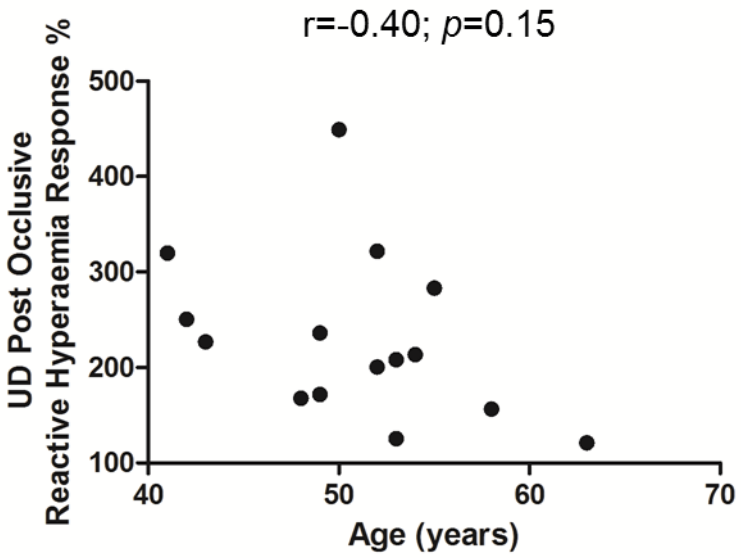


Figure 4.8 The relationship between age and PORH response in Group 2 measured with FLPI using an upper arm cuff and the distal measurement site (*n*=15).

On removal of this subject from the G2 dataset, a significant negative correlation between age and the UP PORH response was found within the modified G2 subject group (r=-0.60, p=0.024, r^2=0.36) (Figure 4.9). This relationship between age and PORH just fell short of reaching statistical significance with the UD PORH response (r=-0.52, p=0.06, r^2=0.27) (Figure 4.10).

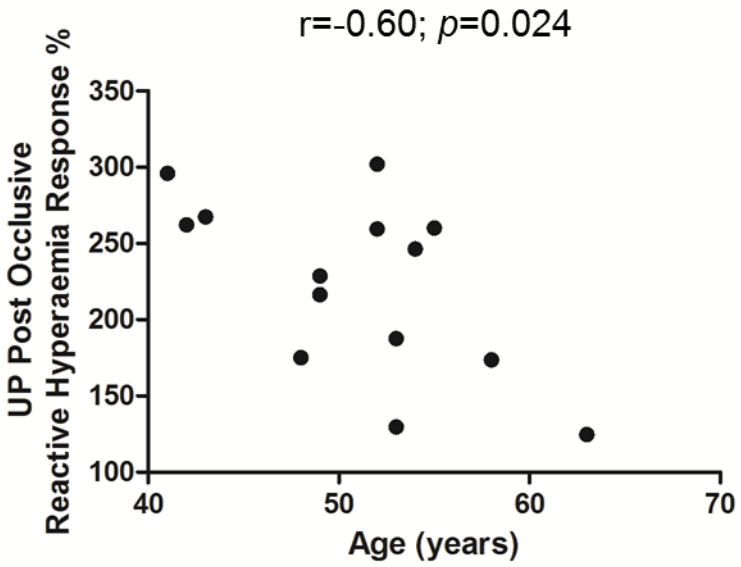


Figure 4.9 The relationship between age and PORH response in Group 2 measured with FLPI using an upper arm cuff and the proximal measurement site after removal of one study volunteer (*n*=14).

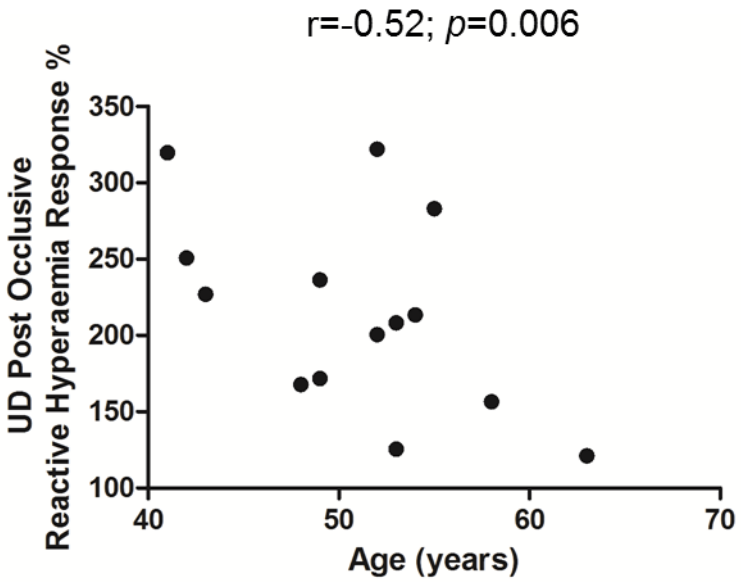


Figure 4.10 The relationship between age and PORH response in Group 2 measured with FLPI using an upper arm cuff and a distal measurement site after removal of one study volunteer (*n*=14).

There was not a significant correlation between age and RHI, AIx or AIx@75 within either G1 or G2 (G1 RHI r=-0.31, p=0.26, r^2=0.01; AIx r=0.21, p=0.44, r^2=0.05; AIx@75 r=0.18, p=0.53, r^2=0.03 and G2 RHI r=-0.20, p=0.45, r^2=0.04; AIx r=0.10, p=0.72, r^2=0.01; AIx@75 r=0.14, p=0.627, r^2=0.02).

4.2.4 Gender Differences

As well as age differences, gender differences were also studied by combining male and female subjects from G1 and G2. Females displayed significantly lower SBP (Females 110.3±8.1 mmHg VS. Males 124.2±13.4 mmHg, $p<0.001$) and lower mean arterial pressure (Females 80.9±7.4 mmHg VS. Males 88.4±9.5 mmHg, $p=0.012$) compared to males. DBP was close to being significantly lower in females than males (Females 66.3±7.8 mmHg VS. Males 70.5±8.2 mmHg, $p=0.08$).

The PORH response measured using the UD set up was significantly higher in females from G1 and G2 compared to males from G1 and G2 (Females 256.9±85.8 % VS. Males 202.1±63.5 %, $p=0.028$). This gender difference in PORH response was not found to be significant with the UP set up (Females 257.2±78.9 % VS. Males 221.0±77.1 %, $p=0.11$). There were no significant differences in the THR between males and females for either UP or UD set ups, however there was a trend towards statistical significance for the UD set up (Females 17810±4383 units VS. Males 15463±3399 units, $p=0.06$).

There were no significant differences in RHI, AIx or AIx@75 between males and females (RHI: Females 2.46±0.5 units VS. Males 2.51±0.7 units, $p=0.41$, AIx: Females 2.7±13.1 % VS. Males 2.5±17.6 %, $p=0.22$, AIx@75: Females -4.9±10.6 % VS. Males 9.3±15.3 %, $p=0.18$). There was also no statistically significant difference between males and females for the uncorrected PAT ratio (Females 3.75±1.0 units VS. Males 3.55±1.9 units, $p=0.35$).

Chapter 5

The Relationship between Skin Microvascular Function and Brachial Artery Velocity Revisited

Analysis performed on data from past research studies within the Vascular and Inflammatory Diseases Research Unit, University of Dundee has shown a relationship between skin microvascular function and FMD velocity; a positive correlation was found between these parameters indicating that better skin microvascular function is associated with a greater FMD velocity. This is an important finding as it is the velocity component of FMD that provides the stimulus for the overall FMD response. The following chapter will explore if this relationship is seen when skin microvascular function is assessed by PORH with FLPI, rather than iontophoresis of the endothelium-dependent vasodilator ACh.

5.1 Methods

PORH was performed on 10 healthy volunteers (7 female and 3 male (mean (±SD) age 26.5 (± 4.6), height 165.4 (± 0.1) centimetres and weight 67.1 (± 5.1) kilograms) at a single visit. The same lower arm cuff PORH protocol was used as described in section 3.1.1; skin perfusion was monitored at the distal measurement site of the forearm throughout the test by FLPI. Alongside PORH, the brachial artery was imaged to collect brachial artery velocities at baseline and following cuff release as described in section

2.1.4. A lower arm cuff position was used instead of an upper arm cuff position so that skin perfusion measurements and brachial artery velocity measurements could be performed simultaneously.

5.1.1 Statistical Analysis

A Pearson correlation was selected for statistical analyses. For all statistical tests a *p* value of <0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS 18 (SPSS Inc., Illinois, Chicago, USA).

5.2 Results

5.2.1 The Relationship between Baseline Skin Perfusion and Baseline Velocities

Skin perfusion at the distal site of the forearm and the brachial artery pulse wave spectral Doppler were investigated to determine whether there was a relationship between the skin microcirculation and brachial artery velocity (integral and maximum) at baseline (pre-occlusion) and hyperaemia (on cuff deflation) (Figure 5.1). The sample size is 9 rather than 10 for both integral and maximum velocities as for each parameter 1 subject's velocity data could not be analysed by the software.

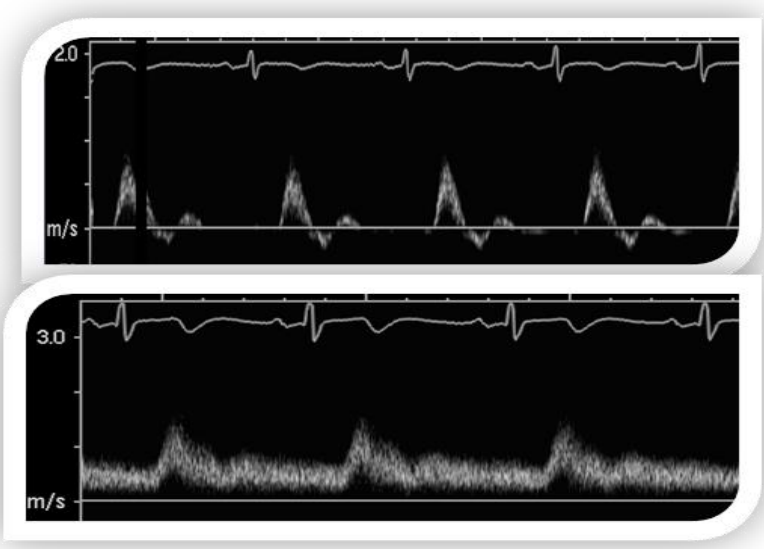


Figure 5.1 Flow mediated dilatation baseline velocity tracing (pre-occlusion) (top) and hyperaemic velocity (post cuff deflation) (bottom) from the Vascular and Inflammatory Diseases Research Unit Laboratory, University of Dundee.

There was a significant negative correlation between skin perfusion and the velocity time integral at baseline (r=-0.69, p=0.04, r^2=0.47) (Figure 5.2). There was not a significant correlation between baseline skin perfusion and the maximum velocity, but there was a trend towards a relationship (r=-0.58, p=0.10, r^2=0.34) (Figure 5.3). The lack of statistical significance for this result is likely due to the small number of subjects investigated rather than a genuine lack of association.

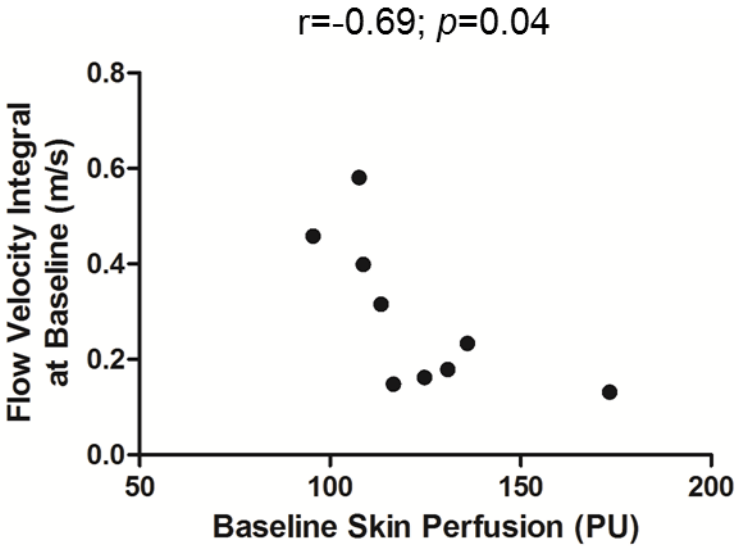


Figure 5.2 The relationship between baseline skin perfusion and flow velocity integral at baseline using a lower arm cuff and a distal measurement site (n=9).

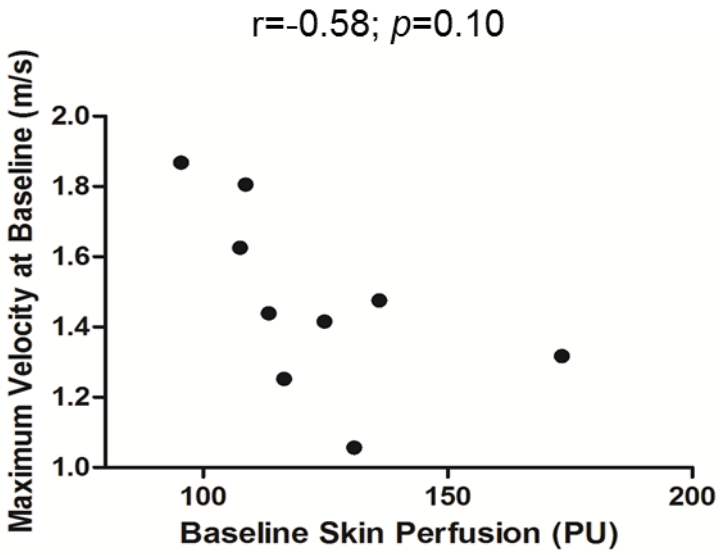


Figure 5.3 The relationship between baseline skin perfusion and maximum velocity at baseline using a lower arm cuff and a distal measurement site (*n*=9).

5.2.2 The Relationship between Peak Skin Perfusion and Peak Velocities

There was not a significant correlation between peak skin perfusion and maximum hyperaemic velocity following a 5 minute cuff occlusion (r=-0.61, *p*=0.08, r^2=0.37) (Figure 5.4). There was a trend towards a significant relationship, but again this may be explained by the small sample size studied. There was a negative correlation between peak skin perfusion and maximum hyperaemic velocity, but this relationship did not reach statistical significance (r=-0.57, *p*=0.11, r^2=0.33) (Figure 5.5). The sample size is 9 rather than 10 for both integral and maximum velocities as for each parameter 1 subject's velocity data could not be analysed by the software.

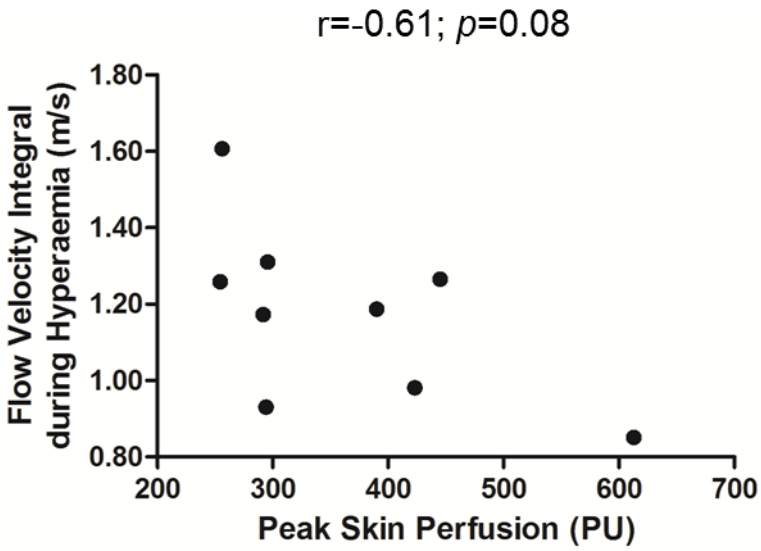


Figure 5.4 The relationship between peak skin perfusion and flow velocity integral during hyperaemia using a lower arm cuff and a distal measurement site (*n*=9).

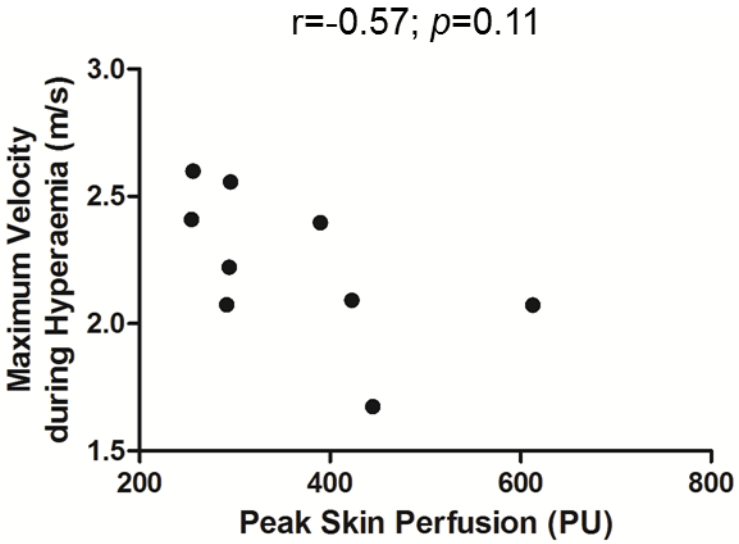


Figure 5.5 The relationship between peak skin perfusion and maximum velocity during hyperaemia using a lower arm cuff and a distal measurement site (*n*=9).

Chapter 6

Discussion

In the current study, a PORH protocol has been developed for use in combination with FLPI to provide an assessment of skin microvascular endothelial function in normal, healthy volunteers free from symptomatic CVD. The developed PORH protocol was then compared with EndoPAT, the only FDA approved measure of endothelial function, to evaluate its ability to detect age related changes in endothelial function between two groups (G1 18-30 years and G2 40-70 years).

6.1 Development of a Post Occlusive Reactive Hyperaemia Protocol with FLPI

6.1.1 Cuff Position and Measurement Site at the Forearm and Reproducibility Testing

It was demonstrated that an upper arm cuff position coupled with a distal measurement site at the forearm was the most reproducible measurement protocol for the assessment of microvascular endothelial function, compared to an upper arm cuff position with a proximal measurement site and a lower arm cuff position with a distal measurement site.

For the assessment of forearm skin blood flow, PORH is most commonly performed with a cuff placed around the upper arm (Roustit and Cracowski, 2013), but a lower arm cuff can also be used and may be preferred by patients as it causes less discomfort than

upper arm cuff occlusion (Mannion et al., 1998). The use of an upper arm blood pressure cuff for a PORH test usually produces a greater hyperaemic response compared with a lower arm cuff position, possibly caused by an increase in the number of resistance vessels recruited in the forearm downstream of the arterial occlusion, leading to a greater flow stimulus (Mannion et al., 1998, Vogel et al., 2000). This was true for visit 2 of the development phase of the PORH protocol, where a greater PORH response was seen using an upper arm cuff occlusion compared to a lower arm occlusion. However at visit 1 of the development phase of the PORH protocol, the lower arm cuff generated a higher PORH response than the upper arm cuff. On cuff release following an upper arm cuff occlusion blood flow may be higher in the deeper tissues, for example muscle rather than the skin, as there is a greater area of tissue (and muscle mass) to revascularise compared with a lower arm cuff. After lower arm cuff occlusion, more blood may flow to the skin as there is less muscle mass at the distal part of the forearm. FLPI predominantly measures blood flow in the superficial, nutritional blood vessels of the skin up to a depth of approximately 300µm, and is therefore unable to detect blood flow responses in the deeper tissues, which may have occurred at visit 1 using the upper arm cuff position.

Skin blood flow displays high spatial heterogeneity; one of the advantages of FLPI is that a specific region of interest can be chosen and blood flow can be averaged over this area, reducing variability. Two measurement sites (proximal and distal) were investigated to look at regional differences in PORH response at the forearm. There was not much difference in the mean PORH responses between visit 1 and visit 2 with an upper arm cuff and the proximal measurement site or an upper arm cuff and the distal measurement site. However, the percentage variability for measurements repeated on two different occasions at the proximal measurement site was almost double that of the distal measurement site; 17.4% versus 8.8%. The differences in variability for the two

measurement sites used in the current study highlight the importance of skin site in microvascular reactivity testing, particularly PORH, due to the heterogeneous PORH responses at the proximal and distal measurement sites at the forearm. FLPI can reduce the variability of skin blood flow through the use of regions of interest at set locations on the skin, and has been shown to have considerably better reproducibility for reactivity tests compared to LDF and LDI techniques (Roustit et al., 2010), but it is unable to completely eliminate regional differences between measurement sites, in this case proximal and distal measurement sites at the forearm.

The PORH response as measured by LSCI has previously been shown to have excellent reproducibility; an 8% coefficient of variation was calculated for the peak cutaneous vascular conductance (CVC), calculated as the ratio of skin perfusion to mean arterial pressure, for two visits, 7 days apart, at the forearm (Roustit et al., 2010). The Roustit study had a similar sample size to the current study (n=14) for assessment of PORH reproducibility using LSCI. His study does not specify the exact position of the region of interest on the forearm so it is not clear if a similar location to either the proximal or distal measurement sites in the present study was used. Furthermore, it is important to mention that the imager used in the Roustit study was not the same as the present study; he used the PeriCam PSI System, Järfalla, Sweden to collect perfusion measurements, an alternative device to FLPI which also uses LSCI technology. It is therefore difficult to make a direct comparison between the current study and the Roustit study owing to these protocol differences.

LDF, LDI and LSCI techniques have previously been compared to assess the reproducibility of the local thermal hyperaemia peak response (Roustit et al., 2010). The coefficient of variation for each method was 40%, 39% and 15% respectively,

additional evidence that the LSCI method used by FLPI has considerably better reproducibility than existing laser Doppler systems.

The PORH response at the distal site was higher at visits 1 and 2 compared to the proximal measurement site. At the distal site on the forearm there is less muscle mass so the perfusion levels may be higher in the skin compared to the proximal site. Here perfusion may be higher in the deeper tissues, like muscle, rather than the skin microcirculation. For the lower arm cuff position there was much greater variation in the PORH response between the two reproducibility visits compared to the upper arm cuff position measurement protocols. The lower cuff protocol was tested for reproducibility on fewer subjects than the upper cuff protocols, however the individual variations for PORH response between visits were higher than the other measurement protocols performed using an upper am cuff occlusion.

The results from this part of the study indicate that an upper arm cuff occlusion and a distal measurement site at the forearm provided the most reproducible assessment of microvascular endothelial function using the PORH test. This is in keeping with current practice where an upper arm cuff is most commonly used for PORH assessments. However if patients find the upper arm cuff occlusion uncomfortable a lower cuff position, which is more tolerable, could be used as an alternative method for PORH testing and assessment of skin reactivity.

6.1.2 Effects of Forearm Skin Temperature on Skin Perfusion and Post Occlusive Reactive Hyperaemia

In this study skin heating was first used to assess baseline skin blood flow at the distal measurement site (the most reproducible site) to standardise skin temperature between subjects. Heating the distal forearm to a temperature of 35°C had no significant effect on skin perfusion at baseline. These results are in agreement with Beed et al., who also

found no significant difference in skin blood flow after heating the skin to 35°C. It was important to look at baseline perfusion with heating before performing the full PORH protocol to assess whether local heating at the distal measurement site initiated a vasodilator response prior to PORH.

When local heating at the distal measurement site was performed in combination with the PORH protocol there was a greater percentage variation between two visits compared to when the PORH test was done without any skin heating (41.2% and 8.8% respectively). Roustit et al. have previously shown an improvement in the reproducibility of PORH at the forearm when baseline skin temperature is standardised. However the improvement in reproducibility of the PORH response was assessed using a single point LDF system rather than the LSCI technique used by FLPI in the current study.

A similar investigation has previously been performed to assess the inter-day reproducibility of PORH using LSCI on healthy volunteers with a seven day interval (Roustit et al., 2010). This study standardised baseline skin temperature prior to the PORH test and found very good reproducibility (8% coefficient of variation) for the peak CVC. The main difference highlighted between the current study and the Roustit study is the device used to assess skin perfusion. Further differences in the PORH protocol used by Roustit and the one used in the current study include expression of results, acclimatisation time, skin temperature and skin site. These parameters were not comparable and therefore the reproducibility results of the current study cannot be directly compared to those from the Roustit study. Although both machines use LSCI technology, the skin perfusion data collected may not be comparable due to differences in device settings, calibration, and skin perfusion signals, resulting in quite dissimilar results.

When baseline skin temperature was standardised to 35°C the PORH responses were lower compared to the responses when there was no standardisation of skin temperature. It is possible that with skin heating there was an increase in perfusion in the deeper tissues prior to hyperaemia that could not be detected by FLPI due to its shallow measurement depth. On cuff release, perfusion may have been directed to the deeper tissues and therefore the PORH response in the skin was blunted compared to when the test was performed without skin heating.

Skin heating was introduced to the PORH protocol to standardise skin temperature between subjects, as it has previously been shown that skin temperature can affect forearm microvascular responses (Roustit et al., 2010). However, heating the skin to a temperature of 35°C failed to improve the reproducibility of the PORH test in the current study. Baseline skin perfusion is influenced by skin temperature; standardising skin temperature through skin heating is one way that initial temperature differences between subjects can be eliminated, however expressing PORH results as a factor of baseline perfusion, as relative values, is an alternative way of correcting the effect of skin temperature.

For the assessment of microvascular endothelial function where FLPI or other laser systems are used, standardisation of protocols is needed. Recommendations have been made for LSCI the technique used by FLPI, for PORH but these focus only on protocol duration for each stage of the test (baseline, occlusion and post cuff recordings) (Mahe et al., 2012). The guidelines fail to include details of cuff position, measurement site or skin temperature. The results from the validation part of the study would propose an upper arm cuff and a distal measurement site at the forearm ($20mm^2$), without skin heating, as the optimal conditions for PORH in the upper limb.

6.2 Finalised Post Occlusive Reactive Hyperaemia Protocol with Full Field Laser Perfusion Imager and Peripheral Arterial Tonometry

6.2.1 Differences between the Groups: G1 VS. G2

BP was higher in G2 for systolic, diastolic and mean arterial BP compared to G1. With age, BP increases from the start of childhood right through to adulthood (Kannel and Gordan, 1978, Whelton, 1985). The increase in BP is a consequence of structural changes to the arteries, namely arterial stiffness and vascular resistance. An increase in large artery stiffness can cause the reflected pressure wave from the arterioles to occur earlier during systole, leading to increases in central BP and pulse pressure. An increase in peripheral vascular resistance in the small vessels contributes to a rise in both systolic and diastolic BPs. An increase in BP with age can also be related to other pathophysiological factors including a reduction in baroreceptor responsiveness and an increase in sensitivity to stimuli of the sympathetic nervous system (Pinto, 2007).

For both groups resting heart rate fell within the typical adult resting heart rate range, although the heart rate in G2 was significantly lower than G1. Under resting conditions, the parasympathetic nervous system dominates and sympathetic activity is suppressed. In G1 perhaps the sympathetic activity was not reduced to the same extent as G2, which resulted in a higher resting heart rate.

Despite the clear differences in BP and heart rate between the two groups, there were no differences in PORH measured using FLPI. A previous study (Hagisawa et al., 1991) investigated the effect of age on PORH in younger and older healthy subjects and, in contrast to the current study, found a significantly lower PORH response in the older group compared with the younger subjects. However there were several differences in study design between the two studies which may account for the variation in results. In the study by Hagisawa et al. there was a greater difference in age between the young

and older groups, an LDF system was used to measure perfusion rather than a LSCI system, a shorter occlusion time was used and ischaemia was induced by applying loads rather than a cuff occlusion. Instead of using a BP cuff, occlusion was achieved by applying two different loads (22.3 N and 44.5 N) to the skin surface using a cylindrical indenter. The load was maintained for 3 minutes to maintain occlusion.

In another study PORH responses assessed using LDF were compared between three groups; a young group, an older sedentary group and an older fit group (Tew et al., 2010). The PORH response was found to be higher in the younger group compared to the older sedentary group in agreement with Hagisawa et al. Of interest was the older fit group, who displayed a considerably higher PORH response than the older sedentary group, suggesting older individuals who maintain a high level of aerobic fitness are able to preserve microvascular function and prevent the age related decline in endothelial function observed in the study by Hagisawa et al. The fitness levels of the subjects in the present study were not considered, but this could be a possible reason why there was not a difference in PORH response between G1 and G2.

The age difference between G1 and G2 may not have been big enough to detect differences in microvascular endothelial function between the two groups. The oldest subjects from G1 and the youngest subjects from G2 may have displayed similar responses to PORH, making it difficult to identify a change in vascular function between the two groups.

Although there were no differences in PORH or THR between G1 and G2, there were significant differences between the groups for resting blood flow. These results are in agreement with some previous studies which have also reported an age related increase in resting baseline skin perfusion (Bari et al., 2005, Ogrin et al., 2005). However, other studies have shown age to have no effect on baseline skin perfusion (Hagisawa et al.,

1991, Tew et al., 2010), while some authors have found a decrease in baseline skin perfusion with ageing (Van den Brande et al., 1997). The differences in results between these studies may be due to the different age groups used in the investigations and the skin site used for examination. A higher baseline blood flow in G2 could be explained by the lower resting heart rate found in G2; a reduction in sympathetic activity would promote less vasoconstrictor tone and therefore could explain the elevated baseline blood flow compared to G1. The higher peak blood flow in G2 may in part be caused by the baseline blood flow in this group starting at a higher level than G1.

There were strong positive correlations between all blood flow parameters obtained from the proximal and distal measurement sites of the forearm. However, reproducibility is not the same at both sites, highlighting the importance of assessing this formally in this study. This relationship is important as it demonstrates that when skin perfusion is assessed using different regions of interest on the forearm similar patterns are observed. Previous skin perfusion experiments conducted using LDF suffered from considerable spatial variability, but the development of systems such as FLPI, which allow perfusion to be measured and averaged over a larger area, provide a means of reducing spatial differences between skin sites.

In contrast to FLPI and the PORH test, there were significant differences between G1 and G2 for all parameters measured by the EndoPAT device; RHI, a measure of endothelial function, and AIx and AIx@75, used to assess arterial stiffness. These parameters were automatically calculated by proprietary software and take into account the control arm, which acts as a control for systemic influences, a major factor when recording blood flow measurements at the finger tips. RHI was higher and AIx and AIx@75 were lower in G1, indicating that younger subjects had better endothelial function and less stiff vessels compared to G2. PAT therefore demonstrated a negative

age related effect on endothelial function and arterial stiffness. G2 had a higher level of arterial stiffness compared to G1, and these results were consistent with BP which was also higher in G2.

A previous study performed EndoPAT on 30 healthy subjects who were divided into two groups of healthy volunteers with similar age groups to the current study (younger subjects mean age 24 years, older subjects mean age 43 years) (Faizi et al., 2009). Although the main aims of this study were to investigate optimum occlusion duration and cuff position, the RHI results were reported for the two age groups. In keeping with the current study, the RHI was found to be lower in the older group compared with the younger group with an occlusion duration of 5 minutes (1.67 units vs. 2.09 units).

The uncorrected PAT ratio was also calculated to assess the PORH response in the occluded arm only, without correction for the control arm. Interestingly there was no significant difference between G1 and G2 when the control arm was omitted from the analysis. The measurement of PORH using FLPI was performed on a single arm without the contralateral arm being used to control for systemic differences. If blood flow measurements had been taken on the opposite arm to PORH, and had been factored into the calculations, differences in PORH response could have potentially been detected between the two groups, as seen with the EndoPAT device.

6.2.2 All Study Data: G1 and G2 (*n*=30)

For the study as a whole, combining G1 and G2 data, age was shown to have a significant positive correlation with systolic and diastolic BP. As mentioned in section 6.2.1, this increase in BP with age is caused by an increase in arterial stiffness and peripheral resistance. The effects of age on BP were investigated in normotensive and hypertensive subjects from the original Framingham study (Franklin et al., 1997). This study found that from age 30 up to 84 years there was a linear increase in systolic BP,

accompanied by an increase in diastolic and mean arterial pressures. However, between ages 50 and 60 there was a decrease in diastolic BP. The increase in all BPs up to the age of 50 is related to a rise in peripheral vascular resistance. The linear increase in systolic BP occurs as a result of an increase in vascular resistance followed by an increase in arterial stiffness in the large arteries. The decline in diastolic BP is likely caused by arterial stiffness playing a more important role than vascular resistance beyond 50 years of age.

There were negative correlations between systolic BP and PORH for each of the measurement sites studied, with a trend towards significance. This study demonstrates that as systolic BP increases the PORH response decreases, indicating a higher systolic BP is associated with poorer endothelial function. It has previously been demonstrated that patients with hypertension have a blunted microvascular endothelial response in the forearm compared to normotensive subjects (Farkas, 2004). The reduction in endothelium-dependent vasodilation associated with hypertension is mainly due to an increase in the production of ROS, resulting in a reduction in the bioavailability of NO (Taddei et al., 2001). This occurs following the activation of a compensatory pathway where cyclooxygenase plays a key role. One possible consequence of a decrease in NO is an increase in endothelin-1, an endothelium derived vasoconstrictor that will promote vasoconstriction when the availability of NO is compromised.

In addition to changes in BP, alterations in endothelial function are also observed with increasing age (Hagisawa et al., 1991, Minson et al., 2002, Holowatz et al., 2003). For the purposes of assessment of endothelial function, changes in blood flow are most commonly performed to identify endothelial dysfunction. A number of studies have found a decline in endothelium-dependent vasodilation with ageing in both the conduit and resistance vessels (Taddei et al., 1995, Singh et al., 2002). This impairment of

endothelial function occurs due to changes in a variety of molecules and processes including a reduction in the activity of eNOS and NO, an increase in the production of ROS and, in turn, oxidative stress, and a decrease in endothelial progenitor cells, leading to a gradual transition from an anti-atherosclerotic endothelial phenotype towards a pro-atherosclerotic state (Torda, 2012, Brandes et al., 2005).

In the present study there was a negative correlation between age and endothelial function, but this relationship only reached statistical significance for RHI calculated from the EndoPAT device. This result supports previous findings that with increasing age there is a reduction in endothelial function.

The two methods used to assess endothelial function were also compared however no significant correlation was found. The weak association may be due to the differences in vascular bed, with EndoPAT assessing the digital pulse waveform in the fingertip, while PORH was performed on the forearm and measured the skin perfusion in the capillaries.

There was a significant positive correlation between age and the arterial stiffness parameters calculated using EndoPAT, AIx and AIx@75, demonstrating an increase in arterial stiffness with advanced age. These results are consistent with other studies where the effect of age on arterial stiffness has been investigated (McEniery et al., 2005, Mitchell et al., 2004). With an increase in arterial stiffness comes several changes, namely luminal enlargement, an increase in wall thickness and a decrease in the elastic properties of the large arteries (Lee and Oh, 2010). In addition, an increase in advanced glycation end products (AGE) and a rise in calcium deposition in the arterial wall also contribute to arterial stiffening (Lee and Oh, 2010).

6.2.3 Within Age Group Correlations

On first analysis, when each age group was studied separately, there was no significant correlation between age and PORH response for either G1 or G2. However, when one subject was removed from the G2 dataset, age and PORH response were found to have a significant correlation in G2. G1 included subjects between the ages of 18 and 30 years, while G2 was made up of subjects aged between 40 and 70 years. The age range of subjects in G1 was narrower than G2 (12 years vs 30 years), and may explain why there was no relationship found between age and PORH at either measurement site for G1. In G2 a broader age range made it easier to detect a change in endothelial function and greater variation in PORH responses between subjects was observed. With advancing age in G2 it was found that the PORH response was lower, indicating a decline in endothelial function with age. The relationship between age and endothelial function was observed for G2 using the UP PORH and UD PORH measurement protocol but only the UP PORH measurement protocol reached significance. Although at the distal measurement site there was only a trend towards a significant correlation between age and PORH, the overall relationship observed at both sites demonstrates similar responses between the two measurement sites at the forearm.

Microvascular endothelial function assessment by EndoPAT failed to identify any age related significant correlations within G1 or G2. The same was true for the arterial stiffness parameters calculated from the EndoPAT device. Interestingly, when G1 and G2 were compared, EndoPAT could detect age related differences between the two groups. These findings indicate that when EndoPAT is used to measure endothelial function (or arterial stiffness) it can detect intergroup differences between two groups made up of different age ranges, G1 vs G2, but it cannot detect intragroup differences when G1 and G2 were looked at individually.

6.2.4 Gender Differences

The effect of gender was also investigated by combining the results from G1 and G2 to study a larger sample size. In the present study females displayed a significantly lower systolic BP and mean arterial BP than their male counterparts, and there was also a trend towards a lower diastolic BP in females. It is well established that at comparable ages BP is higher in males than females (Reckelhoff, 2001) and this pattern continues until women reach the menopause, after which BP rises to levels greater than in males (Reckelhoff, 2001). Oestrogen has been demonstrated to stimulate the production of NO (Weiner et al., 1994) and has been regarded as a cardio-protective mechanism, maintaining BP at lower levels in pre-menopausal women. However a reduction in levels of oestrogen is now only thought to be partly responsible for the change in female BP post menopause, owing to the evidence that hormone replacement therapy does not significantly reduce BP in post-menopausal women (Trial, 1995).

The women recruited into the current study were predominantly pre-menopausal and therefore a lower BP was expected compared to the men. In addition, the higher BP levels found in the male subjects identifies them as having greater CV risk compared to females, in agreement with the consensus that male gender is a CV risk factor in itself.

There was a significant gender difference in PORH using the UD measurement protocol, with females displaying a higher PORH response compared to males. This would suggest that the females recruited into the study have better microvascular endothelial function than the males. It has previously been shown that healthy females have better endothelial function than healthy males of the same age when endothelial function is assessed using FMD (Hashimoto et al., 1995). The gender differences in endothelial function could be partly related to sex hormones and the phase of the female menstrual cycle. An increase in endothelium-dependent vasodilation has been reported

when endothelial function is assessed at the beginning of the luteal phase of the menstrual cycle, due to a concomitant rise in oestrogen levels (Williams et al., 2001). Oestrogen is able to stimulate an increase in eNOS activity, which leads to an increase in NO bioavailability and ultimately an enhanced vasodilator response. The majority of females included in the present study were pre-menopausal and endothelial function could therefore have been affected by the stage of the menstrual cycle. However the stage of the menstrual cycle was not recorded so these findings could not be confirmed.

FMD may not be the best method to assess sex differences in endothelial function; another study reported a higher level of vasodilation in females compared to males, but this difference was shown to be mostly related to the baseline brachial artery diameter rather than the effect of sex hormones (Adams et al., 1996). The assessment of PORH using FLPI is not dependent on vessel size and therefore the difference in endothelial function could be caused by a direct effect of sex hormones.

With ageing, a decline in endothelial function occurs sooner in men than in women (Zeiher et al., 1993), but as women reach the menopause endothelial function deteriorates to levels similar to males of the same age (Celermajer et al., 1994). The males in this study may have had poorer endothelial function compared to the females despite the groups being made up of similar aged subjects. The earlier onset of endothelial dysfunction in males mirrors the trend seen with CVD, where males are seen to develop disease at a younger age compared to females.

In addition to PORH response, the THR was also calculated to assess the time taken for skin perfusion to reach baseline levels. There was a trend towards significance for the UD measurement protocol, with skin perfusion taking considerably longer to return to starting levels in the female group compared to the male group. These results support

the findings that in the present study females displayed better endothelial function in the microvessels than males.

The gender differences in endothelial function identified using FLPI and PORH were not replicated with the EndoPAT device; there were no significant differences in RHI results between males and females. The methods used to assess microvascular endothelial function differ in terms of the vascular bed being investigated. FLPI and PORH evaluated skin microvascular endothelial function at the forearm while EndoPAT looked at microvascular endothelial function in the finger tip. The finger is known to be heavily influenced by the sympathetic and autonomic nervous system, it is possible therefore that other factors were involved which masked the effect gender had on the PAT signal and ultimately the RHI result.

McCue et al. used EndoPAT to assess microvascular endothelial function in a population of 86 healthy adults consisting of 45 males and 41 females with a mean age of 37±5 years. Despite a larger sample size, differences in endothelial function were not detected between males and females using EndoPAT, consistent with the findings when using EndoPAT in the present study.

It is not known whether gender differences would have been revealed if endothelium-independent vasodilation had been evaluated because it was not assessed as part of this study. However, gender differences have previously been identified for endothelium-independent vasodilation using the EndoPAT device (McCue et al., 2012), with females displaying a significantly higher microvascular vasodilation following administration of 0.4 mg of nitro-glycerine when compared to males. The reason for this gender difference is not known, but it may be related to the abundance of sex hormone receptors; females have more oestrogen receptors which may result in a greater sensitivity to vasodilators compared to males (McCue et al., 2012). Although gender

differences have been detected in other studies, the use of nitro-glycerine to assess endothelium-independent vasodilation with EndoPAT is difficult to quantify. This is because endothelium-independent vasodilators act systemically. Therefore the ability to use the contralateral arm as a control to account for autonomic influences during endothelium-dependent vasodilation is lost, as this arm will also be exposed to the endothelium-independent stimulus.

6.3 The Relationship between Pulse Wave Spectral Doppler Recordings and Skin Microcirculation

Recently the brachial artery velocity component of FMD has gained increased interest after it was revealed that during hyperaemia it could be better related to CV risk factors than traditional FMD (Anderson et al., 2011). The velocity pattern provides the stimulus for FMD and is directly related to microvascular function. The brachial artery velocity and forearm skin perfusion were assessed simultaneously at baseline and immediately after 5 minutes of lower arm cuff occlusion to investigate the relationship between these two measures of microvascular function.

6.3.1 The Relationship between Baseline Skin Perfusion and Baseline Velocities

At baseline an inverse relationship was found between forearm skin perfusion and both the integral (AUC) and peak brachial artery velocities. This relationship reached significance for the integral measurement and there was a trend towards significance for the peak velocity.

6.3.2 The Relationship between Peak Skin Perfusion and Peak Velocities

Following cuff release no significant relationships were noted between peak forearm perfusion and hyperaemic integral or peak velocities, but the association did show a trend towards significant of a negative relationship. Interestingly, a lower integral and

peak velocity were related to a higher forearm skin perfusion, and a higher integral and peak velocity were associated with a lower forearm skin perfusion. The results of the present study are surprising as it has previously been reported that a lower hyperaemic velocity is associated with greater CVD risk and is a better predictor of long term CV events in patients with disease than FMD (Huang et al., 2007). It seems strange that a lower hyperaemic velocity was associated with higher levels of skin perfusion during hyperaemia in a group of apparently healthy subjects. These two markers of microvascular function appear to present conflicting results. However it is important to recognise that the counterintuitive results found in the present study may be explained simply by the small number of subjects and in fact if a greater sample size had been studied the results obtained could have been different.

The results from this section of the study do not replicate the relationship between skin microvascular function and FMD in chapter 2 where a positive correlation was found between the ACh AUC and integral velocity. There are two main reasons as to why a different relationship was seen with ACh and PORH, the first of which is the measurement depth used to assess skin microvascular function. LDI, used with iontophoresis of ACh, has a greater measurement depth (1-1.5mm), compared to FLPI (~300μm). A higher brachial artery velocity may have been associated with a higher peak skin perfusion during the PORH test, but there may have been greater blood flow to the muscle rather than the skin. FLPI, which detects superficial skin perfusion, would not have been able to detect this. Secondly, the sample size used to investigate the relationship between PORH and brachial artery velocity was small (n=9) compared to the sample size used to assess ACh AUC and brachial artery velocity (healthy subjects and patients n=266, healthy subjects n=172). The small number of subjects may have affected the relationship between the two parameters and perhaps in a greater sample

size the relationship between PORH and brachial artery velocity may turn out to be positive.

The assessment of FMD relies on a shear stress stimulus created by occluding the forearm microvascular circulation using a pressure cuff. This results in vasodilation of the forearm resistance vessels due to a decrease in vascular resistance. As described in chapter 2, the stimulus for FMD can be influenced by many factors. In particular, the diameter of the brachial artery can determine the size of the shear stress stimulus. The vessel diameter was not considered when comparing the microvascular responses between brachial artery velocities and skin perfusion assessed by PORH in the present study, but this could in part explain the negative association between the two parameters.

6.4 Limitations

One of the limitations identified from the current study is the difference in results depending on whether or not the PAT ratio from the EndoPAT device was corrected for the control arm. EndoPAT only detected a significant difference in endothelial function between G1 and G2 when the control arm was included in the PAT ratio calculation. When the control arm was omitted there was no difference between the groups. FLPI and PORH did not detect differences in endothelial function between the two groups, however no control was used with this method to account for systemic differences. If forearm skin perfusion had been assessed on both arms a calculation could have been performed to incorporate the resting blood flow from the contralateral control arm, providing a PORH response that had been corrected for systemic influences. The small numbers of subjects in each age group, as this was a pilot study, may also have limited the detection of differences in endothelial function between the two age groups.

6.5 Recommendations for Future Work

The current study investigated the microvascular endothelial function in normal, healthy subjects free from symptomatic CVD using FLPI coupled with PORH. Future studies using this method would benefit from an increase in subject sample size which may strengthen the relationships detected in the current study and identify novel findings. Forearm skin perfusion should also be assessed on both arms using FLPI, so that the baseline perfusion in the control arm can be included in the PORH calculation to help eliminate systemic influences. This would provide a representative measure of endothelial function that could be directly compared to the PAT ratio from EndoPAT. In addition, it would be useful for further investigations to be undertaken to assess the developed PORH protocol with FLPI in different patient populations with varying degrees of disease, particularly CVD. This would provide an opportunity to determine if this method is able to distinguish between individuals with varying levels of CVD severity.

6.6 Conclusion

A reproducible PORH protocol has been developed to assess skin microvascular endothelial function in the skin using FLPI. This developed protocol was used to investigate age related changes in endothelial function between two different groups of healthy subjects (18-30 years and 40-70 years) and was compared with the FDA approved EndoPAT device. FLPI coupled with PORH was unable to identify any age related differences in endothelial function between the two age groups, but differences were noted by the EndoPAT device for both endothelial function and arterial stiffness. However a significant negative correlation was found between the PORH response and the older age group (40-70 years) with FLPI. In addition, the PORH test with FLPI did detect differences between males and females, undetected by EndoPAT. The

relationship between endothelial function and age in the older age group and the gender differences identified by FLPI shows that, when combined with PORH, this device offers a promising tool for the assessment of skin microvascular endothelial function and has the potential to identify individuals at risk of developing disease prior to the development of clinical manifestations.

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Appendix A

Participant Information Sheet

Methodological Advances in Non-Invasive Testing of Endothelial Function

Introduction

My name is Fiona Adams and I am a Research Technician and enrolled as a student of Masters by Research at the University of Dundee. As part of my Masters course, I am carrying out a project and I invite you to take part in the following study. However, before you decide to do so, I need to be sure that you understand firstly why I am doing it, and secondly what it would involve if you agreed. I am therefore providing you with the following information. Please read it carefully and be sure to ask any questions you might have and, if you want, discuss it with others including your friends and family. I will do my best to explain the project to you and provide you with any further information you may ask for now or later.

Background

The function of the lining of blood vessels (endothelium) is extremely important in the development and progression of cardiovascular disease (CVD). If damage to the endothelium can be detected early then this will allow early signs of cardiovascular disease to be minimised or reversed. There are many tests currently available to assess endothelial function, but each test comes with its advantages and disadvantages. The aim of this study is to develop a new test which measures the function of the endothelium in normal, healthy volunteers using a novel blood flow monitor (moor Full Field Perfusion Imager (FLPI)). This test will be compared with other already validated measures of endothelial function. We hope this new test of endothelial function will provide a reproducible measure of vascular assessment which has the potential to be used on a larger scale for the prediction of cardiovascular outcome.

What is involved?

We have approached you because we need 30 healthy volunteers to participate in the study. If you are suitable and decide to take part, we will ask you to attend on one or two visit(s) which will last(s) about 1 hour for the first visit and about 1 hour for the second visit, and will be arranged to suit you. The results obtained at the second visit will be used to test the variability of the test results obtained from the first visit.

We will ask you to refrain from food, drink and smoking for at least 2 hours before the tests. At the visit, a test of blood vessel function in your arm will be performed.

Blood flow tests

Reactive hyperaemia This test involves inflating a blood pressure cuff around your upper arm to block the artery for 5 minutes. When the cuff is released, the resulting increase in blood flow in the skin of the forearm (which is related to endothelial function) will be measured using the non-invasive technique of laser Doppler flowmetry. The technique of Laser Doppler flowmetry consists of shining a harmless, low-power laser beam on to the skin and measuring the reflected light. Inflation of the cuff can be uncomfortable and can cause pins and needles, while its release causes a hot flush which some people find unpleasant. However, the test is not anticipated to have any risks to your health.

Skin Heating As part of the standardisation procedure a small area of the skin will be heated. Prior to performing the test of blood vessel function, a small area of skin on the forearm (about the size of a 50 pence coin) will be heated to different temperatures ranging from 30-44 degrees centigrade. The area of skin directly underneath the heater will feel quite warm for a short time.

Finger blood flow (Endothelium Peripheral Arterial Tone (EndoPAT)) We will assess blood circulation in your fingers using a technique called *Endo PAT*. Two probes will be attached to your left and right hand fingers. We will then inflate a blood pressure cuff on one arm for 5 minutes and observe the difference in your blood flow before, during and after we have inflated the cuff.

Risks and benefits

The inflation of a blood pressure cuff around the upper arm or forearm for 5 minutes is uncomfortable and can cause pins and needles, while release of the cuff causes a hot flush as blood returns to the arm, which some people might find unpleasant. There will be no direct benefit to you, but your participation

will help us gain a better understanding to how blood vessels function. We will be happy to reimburse the cost of your travel.

Incidental findings

Since this research involves measuring cardiovascular risk, it is quite possible that it may show up findings other than those under investigation. These are what we call 'incidental findings' the significance of which is often unclear or, indeed, something that the researchers themselves are not qualified to interpret or act upon, if that is the case, expert advice will be sought. It is very important that you understand how 'incidental findings' will be dealt with in this research and you will be asked to give your specific consent to this in the Consent Form that you will sign if you agree to take part. Neither you nor anyone else, including your GP, will be informed of any 'incidental finding' unless the researcher feels that it may have an important bearing on your future health or medical care. If this is the case, such findings will be discussed with you initially and not referred to any other person, including your GP or any relevant hospital specialist without your permission. On the other hand, you are free to instruct the research team that you do not want to be informed of any 'incidental findings' that might arise in the course of the research in which case all such findings will be ignored. It is important that you understand what is intended before you sign the Consent Form, in which case you may wish to discuss this further with a member of the research team or with other independent parties before proceeding.

Your results and confidentiality

The results of this study will be of no direct benefit to you, but will be available at the end on request. Ultimately, we intend to publish them in a professional journal but all information we collect about you, your healthcare records and the results we obtain will remain strictly confidential and anonymised. At no stage will your name, date of birth or your CHI number (Community Health Index number- an identification number that is unique to your medical notes) appear on any dataset for research use. All information will be held on secure databases that are accessible only to staff directly involved in the project. Any record of your name (e.g. on the consent form you sign) and any CHI number record will be held separately from the other information collected and will not be available to researchers. Your personal details will not be given to any third party, nor will it appear in any report or publication that arises from this study.

Complaints and compensation

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. If you believe that you have been harmed in any way by taking part in this study, you have the right to pursue a complaint and seek any resulting compensation through the University of Dundee who are acting as the research sponsor.

Details about this are available from the research team. As a patient of the NHS, you have the right to pursue a complaint through the usual NHS process. To do so, you can submit a written complaint to the Patient Liaison Manager, Complaints Office, Ninewells Hospital (Freephone 0800 027 5507). Note that the NHS has no legal liability for non-negligent harm. However, if you are harmed and this is due to someone's negligence, you may have grounds for a legal action against NHS Tayside but you may have to pay your legal costs.

What do insurance companies think about research?

Participation in this study does not constitute a "genetic test" as defined by insurance companies. The fact that you are taking part in this study should not affect your ability to get insurance. Data will never be released to insurance companies unless we are legally required to do so, and we will strongly appeal this decision

Additional information

It is a requirement that your records in this research, together with any relevant medical records, be made available for scrutiny by monitors from the University of Dundee and NHS Tayside, whose role is to check that research is properly conducted and the interests of those taking part are adequately protected. The Tayside Committee on Medical Research Ethics A, which has responsibility for scrutinising all proposals for medical research on humans in Tayside, has examined the proposal and has raised no objections from the point of view of medical ethics.

Participation in this study is entirely voluntary and you are free to refuse to take part or to withdraw from the study at any time without having to give a reason and without this affecting your future medical care or your relationship with medical staff looking after you.

If you wish to withdraw from this study, identifiable data with consent will be retained and used in the study. No further data will be collected or any research procedures carried out on or in relation to you.

We would be pleased to answer any further questions you might have. Thank you for taking the time to read this information and considering taking part.

Contact

Miss Fiona Adams
f.z.adams@dundee.ac.uk
01382 383479

Dr Faisel Khan
Reader
f.khan@dundee.ac.uk
01382 383531

Appendix B

Consent Form

Methodological Advances in Non-Invasive Testing of Endothelial Function

Dr Faisel Khan and Miss Fiona Adams

Please initial box

1. I confirm that I have read and understand the information sheet dated _____ (version ___) for the above study. I have had the opportunity to consider the information and ask questions, and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time. ☐

3. I agree that I will not be informed of any 'incidental findings' as set out in the Information Sheet unless the researcher decides that such findings may have an important bearing on my future medical care, and I agree to my GP being informed. ☐

4. I agree to take part in the above study. ☐

_____	_____	_____
Name of participant	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Appendix C

Advertisement

Healthy Volunteers Needed

To Take Part in a Study of Blood Vessel Function

This study involves:

- One or Two visits (lasting approximately 1 hour each)
- Non-invasive measurement of blood flow
- Travel expenses provided

Volunteers should be:

- *Healthy and taking no medication*
- *Aged between 18 and 70 years*
- *Without heart and blood vessel problems*

If you might be interested and would like more information, please contact: Miss Fiona Adams, Department of Medicine, Ninewells Hospital

Tel: 01382 496789 Email: f.z.adams@dundee.ac.uk

Appendix D

Ethics Approval

UNIVERSITY OF DUNDEE

School of Psychology

University of Dundee Research Ethics Committee

Fiona Adams,
Division of Medical Sciences
College of Medicine, Dentistry & Nursing,
Ninewells Hospital & Medical School,
Dundee,
DD1 9SY.

5 April 2011

Dear Ms Adams,

Application Number: UREC 11019

Title: Methodological Advances in Non-Invasive Testing of Endothelial Function.

Your application has been reviewed by the University Research Ethics Committee, and there are no ethical concerns with the proposed research. I am pleased to confirm that the above application has now been approved.

You submitted the following documents:

1. research_human_participants_form_completed	2. Consent form updated
3. Information Sheet	4. Additional Notes q11 updated
5. Consent form updated (amended)	6. Hermes advertisement
7. Poster updated	8. research_human_participants_form _updated
9. Participant Information Sheet updated	

Yours sincerely,

Peter Willatts

Digitally signed by Peter Willatts
DN: cn=Peter Willatts, o=University of Dundee, ou=School of Psychology, email=p.willatts@dundee.ac.uk, c=GB
Reason: I am the author of this document
Date: 2011.04.05 11:43:59 +01'00'

Dr Peter Willatts
Chair, University of Dundee Research Ethics Committee

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